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Application No.:	10/5825,705	Examiner:	Sitton, Jehanne Souaya
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Title:	METHOD AND NUCLEIC ACIDS FOR THE IMPROVED TREATMENT OF BREAST CELL PROLIFERATION DISORDERS.		

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
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DECLARATION UNDER 37 C.F.R. § 1.132

Dear Sir:

I, Dr. Jurgen Distler, declare and state that:

1. I have worked at Epigenomics AG for 12 years and currently am Vice President of Product Development based in Berlin, Germany.

2. I received a Ph.D. from Technical University Darmstadt in 1988. I have over 20 years of experience in the field of Molecular Biology and over 12 years experience specifically studying epigenetics and DNA methylation.

3. I am familiar with the prosecution history of U.S. Patent Application Serial No. 10/5825,705.

4. I understand that claims 1-8 and 11-16 are rejected under 35 U.S.C. §112 as allegedly failing to comply with the enablement requirement.

5. I have read and understand "Multicenter Study Using Paraffin-Embedded Tumor Tissue Testing *PITX2* DNA Methylation As a Marker for Outcome Prediction in Tamoxifen-

Treated, Node, Negative Breast Cancer Patients" J. Clin Oncol. 26(31):5036 (2008) (Exhibit A). The data from this study correlated increased DNA methylation of PITX2 with a shorter time to distant metastasis (TDM). (Fig 1, Exhibit A p. 5038). The data further demonstrated a correlation with node negative, hormone receptor positive patients treated with Tamoxifen and higher levels of PITX2 methylation with a shorter TDM. (Fig 1, Exhibit A p. 5039). From this data, I conclude that this article shows that PITX2 DNA methylation was used as a biomarker for outcome prediction in Tamoxifen treated, node negative breast cancer patients allowing for the prediction of low-risk patients who may be treated with Tamoxifen alone.

6. I have read and understand "DNA methylation of the homeodomain transcription factor PITX2 reliably predicts risk of distant disease recurrence in tamoxifen-treated, node negative breast cancer patients- Technical and clinical validation in a multi-center setting in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC) PathoBiology group" Eur. J. Cancer. 43:1679 (2008) (Exhibit B). The data from this study demonstrated that hypermethylation of the PITX2 gene was associated with poor clinical outcome in breast cancer patients, having a hazard score of 2.1. (Tables 2A and 2B, Exhibit B p. 1682). Further, this study defined a low risk group for Tamoxifen therapy alone, of which 86% of breast cancer patients were metastasis free at 10 years post surgery, using a median methylation ratio of PITX2 as the predictor. (Table 2C, Exhibit B p. 1683). From this data, I conclude that this article shows that DNA methylation of PITX2 predicts the risk of distant disease recurrence (metastases) in breast cancer patients.

7. I have read and understand "DNA Methylation Markers Predict Outcome in Node-Positive, Estrogen Receptor-Positive Breast Cancer with Adjuvant Anthracycline-Based Chemotherapy" Clin. Can. Res., 15(1):315 (2009) (Exhibit C). The data from this study demonstrated that hypermethylation of PITX2 is associated with poor prognosis and is independent of traditional predictors of poor prognosis such as age, menopausal status, tumor size, tumor grade, estrogen and progesterone receptors levels. (Table 2, Exhibit C). In addition,

the data implied that methylated PITX2 was a biomarker for more aggressive breast tumors. From this data I conclude that this article shows that PITX2 DNA methylation is an independent predictor of poor prognosis and may be a marker for aggressive breast tumors.

8. I have read and understand "DNA hypermethylation of PITX2 is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients" Breast Can. Res. Treat., 111(3):424-37 (2008) (Exhibit D). The data from this study demonstrated that PITX2 hypermethylation is associated with a high risk of recurrence in node negative, hormone receptor positive breast cancer patients (Table 1, Exhibit D). Figure 1 of Exhibit D further shows that hypermethylation of PITX2 is associated with a shorter TDM in node negative, hormone receptor positive breast cancer patients. From this data, I conclude that this article provides support that PITX2 DNA methylation is a marker for disease recurrence and progression in node negative, steroid hormone receptor-positive breast cancer patients.

9. I have read and understand "Prognostic significance of methylated RASS1A and PITX2 genes in blood and bone marrow plasma of breast cancer patients" Breast Can. Res. Treat., DOI: 10.1007/s10549-010-1335-8 (2011) (Exhibit E). The data from this study demonstrated that hypermethylation of PITX2, isolated from peripheral blood and bone marrow samples, was associated with poor disease free survival and overall survival of breast cancer patients which have received hormone therapy. (Fig. 1 and Table 3, Exhibit E). From this data, I conclude that this article shows that PITX2 DNA methylation in samples of blood and bone marrow plasma of breast cancer patients correlated with a decrease in overall survival and distant disease free survival.

10. I have read and understand "DNA methylation as a biomarker in breast cancer" Future Oncol., 5(8):1245 (2009) (Exhibit F). This review provides evidence that hypermethylation of PITX2 in hormone receptor positive, node negative patients treated with Tamoxifen is associated with a shortened period of disease free survival (Exhibit F p. 1250).

Further, the review shows evidence that methylation analysis of PITX2 may aid in predicting the clinical outcome of hormone receptor positive, node negative patients treated with Tamoxifen as well as the identification of low risk patients who may be sufficiently treated by endocrine therapy alone. (Exhibit F p.1250). Additionally, the review provides evidence that the hypermethylation of PITX2 is associated with aggressive breast cancer and may serve as a biomarker for identifying such tumors. From this data, I conclude that this article shows that PITX2 DNA methylation is a marker for assessment of prognosis or prediction of a therapeutic response in patients with breast cancer. The marker was also used to show outcome predictions in breast cancer patients treated with tamoxifen therapy as well as a biomarker to direct clinical decisions regarding therapy and used as a biomarker for aggressive breast tumors.

11. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

19th Sep 2011
Date

Dr. Jurgen Distler

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ORIGINAL REPORT

Multicenter Study Using Paraffin-Embedded Tumor Tissue Testing *PITX2* DNA Methylation As a Marker for Outcome Prediction in Tamoxifen-Treated, Node-Negative Breast Cancer Patients

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Purpose
We recently reported DNA methylation of the paired-like homeodomain transcription factor 2 (*PITX2*) gene to be strongly correlated with increased risk of recurrence in node-negative, hormone receptor–positive, tamoxifen-treated breast cancer patients using fresh frozen specimens. Aims of the present study were to establish determination of *PITX2* methylation for routine analysis in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue and to test *PITX2* DNA methylation as a biomarker for outcome prediction in an independent patient cohort.

Patients and Methods
Real-time polymerase chain reaction (PCR) technology was validated for FFPE tissue by comparing methylation measurements in FFPE specimens with those in fresh frozen specimens from the same tumor. The impact of *PITX2* methylation on time to distant metastasis was then evaluated in FFPE specimens from hormone receptor–positive, node-negative breast cancer patients (n = 399, adjuvant tamoxifen monotherapy).

Results
Reproducibility of the PCR assay in replicate measurements ($r_s \geq 0.95$; n = 150) and concordant measurements between fresh frozen and FFPE tissues ($r_s = 0.81$; n = 89) were demonstrated. In a multivariate model, *PITX2* methylation added significant information (hazard ratio = 2.35; 95% CI, 1.20 to 4.60) to established prognostic factors (tumor size, grade, and age).

Conclusion
PITX2 methylation can be reliably assessed by real-time PCR technology in FFPE tissue. Together with our earlier studies, we have accumulated substantial evidence that *PITX2* methylation analysis holds promise as a practical assay for routine clinical use to predict outcome in node-negative, tamoxifen-treated breast cancer, which might allow, based on future validation studies, the identification of low-risk patients who may be treated by tamoxifen alone.

J Clin Oncol 26:5036-5042. © 2008 by American Society of Clinical Oncology

INTRODUCTION

Current guidelines recommend adjuvant chemotherapy followed by endocrine therapy for a substantial number of patients with node-negative, steroid hormone receptor–positive breast cancer.[1,2] Yet, most of these patients have a rather good prognosis and would derive sufficient benefit from endocrine treatment alone, suggesting that they could be spared potential overtreatment by adjuvant chemotherapy. Unfortunately, such low-risk patients cannot be reliably identified by using only traditional prognostic factors such as tumor size, grade, or age.[3]

DNA methylation of cytosine phosphoguanine dinucleotides within gene regulatory regions is a common and early event in cancer.[4,5] Frequently, hypermethylation within gene promoter regions is associated with suppression of gene expression.[6] We have recently correlated DNA methylation of 117 candidate genes with outcome of breast cancer patients after adjuvant tamoxifen therapy. Among all genes analyzed, DNA methylation of the paired-like homeodomain transcription factor 2 (*PITX2*) gene showed the strongest association with metastasis-free survival and was selected for further analysis.[7] *PITX2* is required for morphogenesis of anterior

structures, such as eyes, teeth, and anterior pituitary, and plays a role in left/right patterning.[8] Regulation by the WNT/DVL/beta-catenin and hedgehog/transforming growth factor β pathways[9] and differential expression in pituitary adenomas were reported.[10-12] In our previous study, into which *PITX2* had been included based on reported methylation of the gene in acute myeloid leukemia,[13] a potential link between *PITX2* and breast cancer was established.[7]

In the present study, we aimed to test the clinical relevance of *PITX2* as observed in fresh frozen breast cancer specimens[7] for formalin-fixed paraffin-embedded (FFPE) tissue. For this purpose, a real-time polymerase chain reaction (PCR)–based quantitative DNA methylation assay[7] was adapted for FFPE breast cancer specimens. We then used this assay to analyze the DNA methylation score of *PITX2* in FFPE tissue obtained from 427 patients with steroid hormone receptor–positive, node-negative primary breast cancer treated by adjuvant tamoxifen alone. None of these patients had been included in the previous study.[7] Our results substantiate the evidence that *PITX2* DNA methylation may be a clinically relevant biomarker for outcome prediction in hormone receptor–positive, node-negative breast cancer.

PATIENTS AND METHODS

Technical Assay Validation

Paraffin blocks from 30 breast cancer patients were obtained from a commercial provider and used to establish the analytic performance of the *PITX2* quantitative methylation PCR (QM-PCR) assay. From each block, 15 sections (10 μm) were prepared. Three consecutive sections were pooled and used for DNA extraction, thus resulting in five different preparations of genomic DNA per tumor block. In addition, 89 pairs of frozen and FFPE specimens obtained from the same tumor were provided by the Department of Obstetrics and Gynecology, Technical University of Munich, Munich, Germany; University Hospital Eppendorf, Hamburg, Germany; and Clinical Experimental Oncology Laboratory, National Cancer Institute, Bari, Italy.

Clincial Testing Study

Consecutive breast cancer patients (n = 427), who had not been part of our previous study,[7] were identified in 10 clinical centers (Department of Obstetrics and Gynecology, Technical University of Munich, Munich; Institute of Pathology, Charite University Hospital, Berlin; Institute of Pathology, University of Regensburg, Regensburg; Department of Visceral, Thoraic, and Vascular Surgery, University Hospital Carl Gustav Carus, Technical University Dresden, Dresden; Department of Gynecology, University Hospital Hamburg Eppendorf, Hamburg, Germany; Institute of Oncology, Ljubljana, Slovenia; Clinical Experimental Oncology Laboratory, National Cancer Institute, Bari, Italy; Halitus Instituto Medico, Buenos Aires, Argentina; Department of Medical Oncology, Erasmus Medical Center, Rotterdam, the Netherlands; and Albany Medical College, Albany NY). Inclusion criteria were availability of FFPE specimens with an invasive tumor component, stage pT1-3 disease, estrogen receptor (ER) and/or progesterone receptor (PgR) expression (either 10 fmol/mg of cytosolic protein determined by enzyme immunoassay or dextran-coated charcoal or positive Remmele score[14]), no pathologic axillary lymph node involvement, age more than 35 years at time of diagnosis, surgery in or before 1998, adjuvant tamoxifen monotherapy (indicated duration of 5 years), availability of follow-up data, and appropriate patient consent. Patients treated using neoadjuvant or adjuvant chemotherapy were excluded. Primary therapy and regular follow-up were performed according to local guidelines. Patient characteristics are listed in Table 1. Approval for the study was obtained from the local ethics committee of each participating center.

Three consecutive tissue sections (10 μm) were prepared for DNA methylation analysis. An additional consecutive section was stained with hematoxylin and eosin and used to confirm presence of invasive cancer and for centralized determination of histopathologic tumor grade by one pathologist (G.K.) according to the Nottingham modification of the Scarff-Bloom-Richardson grading scheme.[15] Human epidermal growth factor receptor 2 (HER-2)/*neu* amplification was assessed by immunohistochemistry using a semiquantitative scoring system or fluorescent in situ hybridization analysis, as described previously.[16]

Table 1. Patient Characteristics

Characteristic	No. of Patients (N = 399)	%
Tumor grade		
1	62	15.5
2	288	72.2
3	36	9.0
Unknown	13	3.3
Tumor size		
< 2 cm (T1)	240	60.2
≥ 2 cm (T2 + T3)	158	39.6
Unknown	1	0.2
Age at time of diagnosis, years		
≤ 50	52	13.0
> 50	347	87.0
Age, years		
Median	63	
Range	34-86	
Steroid hormone receptor status		
ER positive	391	98.0
ER negative	3	0.8
ER unknown	5	1.2
PgR positive	297	74.4
PgR negative	54	13.5
PgR unknown	48	12.0
HER-2/*neu* status*		
Positive	28	8.8
Negative	292	91.2
Follow-up time (TDM),† months		
Median	63	
Range	1-158	

NOTE. Patients with valid methylation measurements were included in the statistical analysis.

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor HER-2, human epidermal growth factor receptor 2; TDM, time to distant metastasis.

*Patients with immunohistochemistry analysis resulting in scores of, 1+ and 2+ (n = 20), together with score 0, were assigned to the HER-2/*neu*-negative group; patients with a score of +3 were assigned to the HER-2/*neu*-positive group.

†In patients without evidence of distant metastasis at time of last follow-up.

DNA Extraction and Bisulfite Conversion

FFPE tissue sections were incubated in limonene (Fluka, Deisenhofen, Germany) for 10 to 30 minutes to remove paraffin. For DNA extraction, the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations (extended lysis for 2 days at 50°C). The DNA Tissue Mini Kit (Qiagen) was used to isolate DNA from fresh frozen tumor specimens. Protocols for bisulfite treatment of genomic DNA isolated from FFPE specimens[17] and fresh frozen specimens[7] were used as previously described.

Quantitative Real-Time PCR

Quantitative real-time PCR (QM-PCR) to assess *PITX2* methylation was conducted as previously described.[7] Primers excluding cytosine phosphoguanine dinucleotides and TaqMan probes specific for methylated and unmethylated states were designed to target the *PITX2* gene promoter region (for details, see Appendix, online only). Threshold cycles (Ct) were used to calculate methylation scores: methylation score = $100/[1 + 2^{*}(Ct_m - Ct_u)]$; Ct_m

and Ct_0 denote threshold cycles of the probes specific for the methylated and unmethylated state, respectively. The median of at least three replicate measurements was calculated for each sample and used for statistical analysis. Predefined quality criteria were set such that measurements with both probes greater than 38 cycles were excluded.

Statistical Analyses

The relationship between *PITX2* DNA methylation score and established clinical factors to the primary end point, time to distant metastasis (TDM), was analyzed using univariate and multivariate Cox proportional hazard models.[18,19] Contralateral disease, other second primary cancers, death before distant recurrence, and locoregional recurrence were considered censoring events. Hazard ratios (HRs) for continuous variables were calculated relative to an increment of the interquartile range (25% to 75% quantile).[19] Wald tests were used to test for significance of HRs. Survival curves were calculated according to the Kaplan-Meier method.[20]

RESULTS

PITX2 DNA Methylation Measurements in FFPE Primary Breast Cancer Tissue

At first, we aimed to verify that DNA methylation measurements can be reliably obtained from routinely archived specimens. For this purpose, replicate measurements of 150 tissue preparations from 30 paraffin blocks originating from different breast cancer patients were performed. We observed high correlation of repeated measurements of DNA isolated from the same tissue preparation (Spearman correlation, $r_s = 0.95$; n = 150; Fig 1A), indicating that *PITX2* DNA methylation scores can be reproducibly assessed using FFPE breast cancer tissue specimens.

Next, we compared 89 fresh frozen and FFPE tissue specimens obtained from the same tumor to determine the impact of different sample processing techniques on DNA methylation measurements and found high concordance regarding *PITX2* DNA methylation scores between fresh frozen and FFPE samples ($r_s = 0.81$). We further explored whether *PITX2* DNA methylation measurements may be affected by tumor tissue heterogeneity by comparing results obtained from different samplings of the same primary tumor block. Five samples were prepared from 30 blocks, each block representing a different primary tumor from a different patient. As shown in Figure 1B, the interindividual differences, assessed by averaging all measurements from the same tumor, were significantly higher (standard deviation, 25.7%; range, 0% to 88%) than measurement differences within individual tumors (average standard deviation, 4.3%; range, 0.3% to 17%). Given these results, we concluded that our workflow and our tissue sampling procedure were well suited to reliably obtain representative *PITX2* DNA methylation results using FFPE specimens.

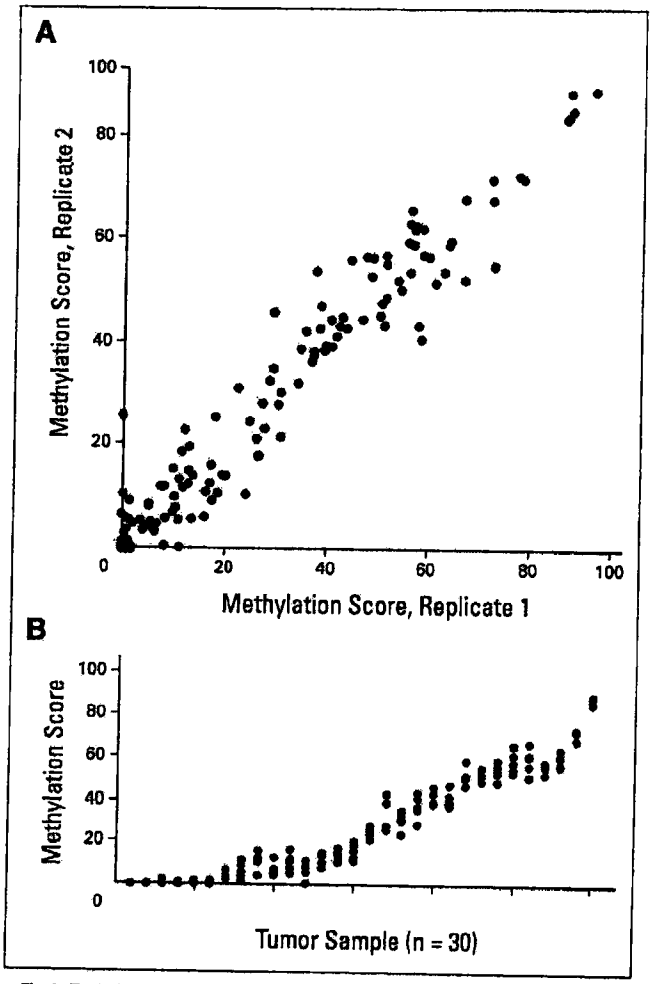

Fig 1. Technical validation of paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation measurements in formalin-fixed paraffin-embedded breast cancer tissue specimens. (A) Repeatability. Quantitative methylation polymerase chain reaction was used to measure *PITX2* DNA methylation scores in 150 DNA preparations from 30 different breast carcinomas in three replicates each. Highly concordant results between replicate measurements were obtained ($r_s \geq 0.95$). (B) Intra- and intersample variability. *PITX2* DNA methylation scores were determined in consecutive preparations (n = 5) from the same paraffin block. Results for 30 different tumors, ranked by the median methylation score for each tumor, are shown. The variability of *PITX2* DNA methylation scores among different tumors, assessed by averaging over all measurements from the same tumor, were significantly higher (standard deviation, 25.7%; range, 0% to 88%) compared with the variability among different preparations from the same tumor (average standard deviation, 4.3%; range 0.3% to 17%).

Clinicial Testing Study

FFPE specimens obtained from 427 primary breast cancer patients from nine clinical centers were analyzed for *PITX2* DNA methylation scores using QM-PCR. All patients included in this retrospective clinical study had steroid hormone receptor–positive, node-negative breast cancers and had received tamoxifen as sole adjuvant systemic therapy. Valid DNA methylation measurements passing the predefined quality control criteria were obtained for 399 patients (93.4%; Table 1).

TDM was defined as the primary end point, and the proportion of patients without evidence of metastasis after 10 years was estimated to be 88.8%. Among the established prognostic factors, only tumor size was a significant predictor of outcome by univariate analysis ($P = .001$; Table 2).

In concordance with our previous model development study,[7] we observed a strong correlation of increased *PITX2* DNA methylation scores with shorter TDM (HR = 2.75; 95% CI, 1.40 to 5.41; Wald test, $P = .004$; Appendix Table A1, online only), whereas correlation with overall survival was not significant (HR = 1.16; 95% CI, 0.67 to 2.02; $P = .59$). To illustrate the potential of *PITX2* DNA methylation as a prognostic test, we used the patient group (n = 236) reported previously[7] as an independent training set, which included,

Table 2. Univariate and Multivariate Analyses for Time to Distant Metastasis of Established Prognostic Factors and *PITX2* DNA Methylation

Variable	Hazard Ratio	95% CI	*P*	No. of Patients
Univariate analysis				
PITX2 DNA methylation	2.75	1.40 to 5.41	.004*	399
Tumor size (T2, T3 *v* T1)	7.92	2.34 to 26.82	.001*	398
Tumor grade (3 *v* 1, 2)	1.00	0.23 to 4.28	.999	386
Age at time of diagnosis	1.74	0.88 to 3.46	.112	399
PgR status (positive *v* negative)	3.52	0.47 to 26.29	.220	351
HER-2/*neu* status (positive *v* negative)	2.60	0.76 to 8.98	.132	320
Multivariate analysis				
PITX2 DNA methylation	2.35	1.20 to 4.60	.013*	385
Tumor size (T2, T3 *v* T1)	6.82	1.98 to 23.5	.002*	385
Tumor grade (3 *v* 1, 2)	0.55	0.13 to 2.42	.43	385
Age at time of diagnosis	1.32	0.61 to 2.86	.48	385

NOTE. Tumor size, grade, progesterone receptor (PgR) status, and human epidermal growth factor receptor 2 (HER-2)/*neu* status were included in the analysis as binary variables. For continuous variables, paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation score and age at time of diagnosis hazard ratios for distant recurrence were calculated relative to an increment of the interquartile range of that variable (ie, for the increment from the 25% quartile to the 75% quartile of the measurements). Wald tests were used to calculate *P* values.
*Statistically significant.

like the patient group in this report, exclusively ER-positive, node-negative patients treated only with tamoxifen. We defined the corresponding median methylation score as the cut point for the present study. Using Kaplan-Meier analysis, we observed a significant risk group separation (log-rank test, $P = .03$), with 52% of the 399 patients classified into the good prognosis group (Fig 2).

To analyze the *PITX2* DNA methylation score as a biomarker in the context of other clinical parameters, Cox multivariate regression analysis was performed including tumor size, grade, age at diagnosis, and *PITX2* DNA methylation score. Complete data were available for 385 patients. *PITX2* DNA methylation score (analyzed as a continuous variable) contributed significant additional information (HR = 2.35; $P = .013$) to the multivariate model (Table 2).

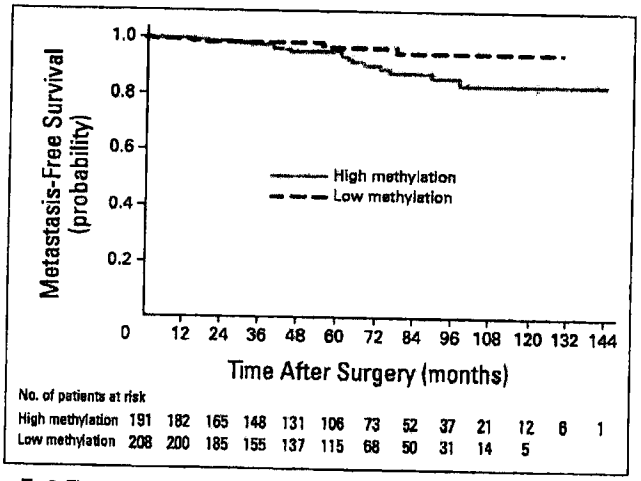

Fig 2. Time to distant metastasis in steroid hormone receptor–positive, node-negative patients (n = 399) treated for 5 years with tamoxifen as their sole adjuvant systemic therapy. Patients were stratified into two groups according to the paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation score as measured by quantitative methylation polymerase chain reaction assay. The methylation score cutoff was derived using the patient group (n = 236) from our previous study[7] as an independent training set. To account for a potential bias in assay performance over time, we compared measurements of 84 patient samples analyzed in both the present and the previous study[7] and determined the numerical value of the cutoff for the present validation study (methylation score = 22.9; see Appendix Fig A2 for details). Statistically significant separation of the two groups was observed (log-rank test, $P = .032$; hazard ratio = 2.69 [*PITX2* dichotomized]; 95% CI, 1.05 to 6.89). Patients in the low-risk group were characterized by *PITX2* methylation scores less than the cutoff.

DISCUSSION

To successfully establish a tumor biomarker for routine clinical use, not only does relevance to clinical management have to be demonstrated, but also the technology to detect a marker needs to be applicable in clinical routine. Aberrant DNA methylation has been well established as an early, frequent, and stable molecular event during tumor progression.[21-28] Using fresh frozen tumor specimens, we previously reported *PITX2* DNA methylation to be a strong marker for outcome prediction in patients with steroid hormone receptor–positive, node-negative primary breast cancer treated with adjuvant tamoxifen monotherapy.[7]

Compared with fresh frozen tissue, nucleic acids isolated from FFPE tissue have been reported to be more often degraded, thus representing potentially less than ideal tissue for molecular analysis.[29] Nevertheless, even on FFPE samples, we were able to demonstrate high repeatability of measurements for our workflow (Fig 1A). Moreover, we observed high correlation of measurements between FFPE and fresh frozen tissue ($r_s = 0.81$) when analyzing matched tissue pairs obtained from the same tumor. Taking into account that fresh frozen and FFPE samples not only differed in preparation techniques but also were subject to tumor heterogeneity within each tumor, we considered this high correlation adequate to exclude systematic differences in methylation measurements between fresh frozen and FFPE specimens. To further explore the impact of tumor heterogeneity on methylation analysis, we determined *PITX2* methylation scores of different samples from the same tumor tissue (Fig 1B). The fact that we found relatively low variability of methylation measurements within individual tumors compared with variability between tumors provides further evidence for the potential of *PITX2* methylation analysis for routine molecular diagnostics. Our robust and reliable workflow for

DNA methylation measurement was further exemplified in our multicenter clinical study, where we obtained successful measurements in 93.4% of the patients (399 of 427 patients) analyzed.

For further clinical testing of the *PITX2* biomarker, 427 FFPE specimens from node-negative, steroid hormone receptor–positive breast cancer patients treated with adjuvant tamoxifen monotherapy were collected from nine clinical centers. Our results confirm the potential of *PITX2* DNA methylation as a marker for risk of recurrence (HR = 2.75; 95% CI, 1.40 to 5.41). We used a previously analyzed patient cohort[7] as a training set to define the methylation score cut point for Kaplan-Meier analysis. Applying this cut point to stratify our study population (n = 399) resulted in a significant risk group separation regarding TDM (P = .03), with 52% of patients being classified as low risk with an estimated 10-year metastasis-free survival rate of 95%. These results suggest that *PITX2* DNA methylation analysis might be used to identify node-negative, steroid hormone receptor–positive breast cancer patients who may be sufficiently treated using adjuvant tamoxifen alone. Moreover, these low-risk patients, if postmenopausal, may not require aromatase inhibitor therapy. For patients in the higher risk group, the additional benefit from aromatase inhibitors and/or adjuvant chemotherapy needs to be evaluated. The relatively late separation of survival curves could suggest that patients with a high *PITX2* methylation score might not derive benefit from the well-established tamoxifen carryover effect.[30] However, whether *PITX2* methylation predicts tamoxifen response or is prognostic independent of therapy cannot be distinguished at present. In this context, it is interesting to mention that *PITX2* DNA methylation was also found to be a strong prognostic factor for distant metastasis–free survival and overall survival in a cohort of 412 primary breast cancer patients enrolled in the Netherlands who did not receive any adjuvant systemic therapy.[31] The latter study suggests that performance of *PITX2* in tamoxifen-treated breast cancer patients might, in part, rely on a prognostic component. In a further study, we found that *PITX2* methylation predicted outcome for distant metastasis–free survival and overall survival in a patient group of 241 ER-positive, node-positive, HER-2/*neu*–negative patients treated with adjuvant chemotherapy (Hartmann O et al, submitted for publication).[32] In our present study, however, we were not able to demonstrate a significant association of *PITX2* DNA methylation with overall survival, likely because of the relatively low number of events in this patient group with good overall prognosis.

We analyzed the clinical impact of *PITX2* DNA methylation taking into account the established prognostic factors of tumor size, grade, and age. In the multivariate model, *PITX2* DNA methylation contributed statistically significant independent outcome information. Univariate analysis showed that only tumor size was significantly associated with outcome, a result that is in line with previous observations.[32] Interestingly, we found different proportions for T stage when comparing our patient training set of 236 patients[7] (35% with stage T1) with our current testing set (60% with stage T1). A possible explanation for this difference could be that the specimens obtained from the patient training group (n = 236) were in large part collected for routine ER and PgR determination by biochemical methods, which, at the time, was not routinely performed for the smaller T1 tumors. Recently, ER status and PgR status have been routinely assessed by immunohistochemistry in FFPE tissue, which is feasible even for the smallest T1 tumors. Furthermore, because the training set patients were diagnosed earlier in time compared with the testing set, over-representation of larger tumors in the training set might also be explained in part by the general trend toward smaller tumors in more recent times, mostly as a result of mammography screening.[33] It seems important to consider that, in our group of patients, most tumors (72%) were classified as grade 2 (moderately differentiated) and that, therefore, the remaining groups of grade 1 and grade 3 tumors were relatively small for meaningful subgroup analysis. Although, in addition to ER status, quantitative measurements of ER levels were only available for a subgroup of patients (n = 286), our finding that the actual ER level is not a significant predictor of TDM (data not shown) in this ER-positive group is in line with reports on similar treatment groups.[34] As is expected for an ER-positive population, only 15% of the patients for whom PgR status was reported were PgR negative. No statistically significant association of PgR status with patient outcome was observed. In our patient group, prognostic significance of HER-2/*neu* status could not be demonstrated (HR = 2.60; 95% CI, 0.75 to 8.98; P = .13) likely because of the small proportion of HER-2/*neu*–positive patients (n = 28; 8.8%); however, this is in line with previous observations in similar patient groups.[35] According to current guidelines,[2] the majority of HER-2/*neu*–positive patients will receive trastuzumab as adjuvant therapy, and only HER-2/*neu*–negative patients qualify for tamoxifen monotherapy. Therefore, it is important to note that *PITX2* methylation predicted outcome in the HER-2/*neu*–negative patient subgroup in univariate (P = .039) and multivariate analyses (P = .019; Appendix Fig A1, online only). Patient age has been associated with risk of disease recurrence.[35] However, in our cohort, the majority of patients were older than 50 years at time of diagnosis, indicating that, at the time our patients were treated for primary breast cancer (≤ 1998), most premenopausal patients did not receive adjuvant tamoxifen. As a consequence, younger patients were not present in significant numbers in our cohort, which may explain the lack of correlation of age with outcome. In summary, we conclude that the distributions of covariates as well as their performance for outcome prediction in our patient cohort are within expectations from similar studies in the literature.

Although our results represent, to our knowledge, the first large-scale testing of a DNA methylation marker for outcome prediction in primary breast cancer, other groups have identified respective gene expression signatures.[36-38] In fresh frozen tumor tissue, prognostic signatures have been reported for breast cancer patients who had not received any adjuvant systemic therapy.[37,38] So far, logistics for preservation of fresh frozen tissue are only established in specialized centers. Moreover, tumor sizes at primary diagnosis are decreasing as a result of improved early detection. Thus, the amount of fresh breast cancer tissue remaining after routine histopathology examination may be limited. Using RNA isolated from FFPE specimens, a recurrence score was developed for tamoxifen-treated patients based on reverse transcription PCR analysis of 21 genes.[36] With regard to establishing a new biomarker for clinical decision making, the key criterion will most likely be the outcome in the low-risk group; the predicted proportion of patients without evidence for distant metastasis will have to be low enough as to safely allow avoiding adjuvant chemotherapy. In addition, this low-risk group has to include a substantial proportion of patients to justify testing the whole population. For both criteria, the recurrence score (low-risk group size, 51%; 10-year metastasis-free survival rate, 93.2%)[36] and our single DNA methylation marker *PITX2* (low-risk group size, 52%; 10-year metastasis-free survival rate, 95%; Fig 2) seem to render comparable results.

Our studies provide strong evidence that, based on measurement of *PITX2* DNA methylation, a relevant proportion of patients with steroid hormone receptor–positive, node-negative breast cancer may be considered to derive sufficient benefit from adjuvant endocrine therapy with tamoxifen alone. The results are encouraging to initiate further large-scale studies in well-characterized patient groups, ultimately aiming at a prospective clinical validation study in a similar set up as described for other breast cancer gene signatures.[39,40] Measurement of a single stable DNA-based marker from FFPE tissue holds promise as a practical, robust, and cost-effective method for routine clinical application.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

EXHIBIT B

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DNA-methylation of the homeodomain transcription factor PITX2 reliably predicts risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients – Technical and clinical validation in a multi-centre setting in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC) PathoBiology group

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ABSTRACT

Our aim was to identify and validate DNA-methylation markers associated with very good outcome in node negative, hormone receptor positive breast cancer patients after adjuvant endocrine therapy which might allow identifying patients who could be spared the burden of adjuvant chemotherapy. Using a methylation microarray, we analysed 117 candidate genes in hormone receptor-positive tumours from 109 breast cancer patients treated by adjuvant tamoxifen. Results were validated in an independent cohort ($n = 236$, 5 centres). Independent methodological validation was achieved by a real-time polymerase chain reaction (PCR)-based technique. DNA methylation of PITX2 showed the strongest correlation with distant recurrence. Its impact on patient outcome was validated in the independent cohort: 86% of patients with low PITX2 methylation were metastasis-free after 10 years, compared to 69% with elevated PITX2 methylation. Moreover, PITX2 methylation added significant independent information to established clinical factors. All clinical and

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technical findings were confirmed by quantitative DNA-methylation PCR. These results provide strong evidence that DNA-methylation analysis allows clinically relevant risk assessment in tamoxifen-treated primary breast cancer. Based on PITX2 methylation, about half of hormone receptor-positive, node-negative breast cancer patients receiving adjuvant tamoxifen monotherapy can be considered low-risk regarding development of distant recurrences and may thus be spared adjuvant chemotherapy. In addition, these low-risk postmenopausal patients seem to respond sufficiently well to tamoxifen so that they may not require up-front aromatase inhibitor therapy.

1. Introduction

Current guidelines recommend adjuvant chemotherapy followed by endocrine therapy for most women with node-negative, steroid hormone receptor-positive breast cancer.[1] This recommendation is based on a significant reduction of the risk of disease recurrence by chemotherapy in this population, independent of the risk reduction by endocrine therapy.[2] However, these patients have a rather good prognosis and generally derive significant benefit from endocrine treatment alone.[2] Hence, after endocrine treatment, the majority will never suffer recurrence and thus would have been adequately treated by tamoxifen alone. Unfortunately, traditional prognostic factors are not adequate to identify those patients at low risk who can be spared over-treatment by chemotherapy, as is true in the majority of hormone receptor-positive, node-negative patients. In postmenopausal hormone receptor-positive patients, aromatase inhibitors have become an important treatment option.[3] Yet, it is still unclear which patients will be sufficiently treated by adjuvant tamoxifen and which will benefit more from aromatase inhibitors – a rather important question given the lack of information on long-term side effects and the increased costs for aromatase inhibitors.

A common and early event in cancer is aberrant DNA methylation of cytosine phosphoguanine dinucleotides (CpG) within gene regulatory regions.[4,5] Frequently, hypermethylation of promoters is associated with suppression of gene expression.[5,6] DNA-methylation patterns are tumour-specific and can be used for molecular subclassification of tumours.[7–9] Additionally, several studies have demonstrated the potential of DNA-methylation as prognostic or predictive markers in a variety of cancers.[10–12] Recently, some studies have suggested that methylation of certain genes may correlate with tamoxifen response and survival in breast cancer.[13,14]

Our aim was to identify and validate, for the first time, DNA-methylation markers associated with a low risk of distant recurrence in breast cancer patients receiving adjuvant tamoxifen monotherapy. Using a previously described microarray approach[7,14], we analysed DNA methylation of 117 candidate genes in primary tumours of 109 hormone receptor-positive breast cancer patients, all of whom had received tamoxifen as their sole adjuvant systemic treatment. The candidate genes were selected because of their potential function in breast tumourigenesis or metastasis, because of their presumed role in resistance to endocrine therapy or steroid hormone regulation, or because they had been described as being methylated in cancer. In the subsequent validation study, 33 candidates were selected and analysed in an independent cohort encompassing 236 node-negative, hormone receptor-positive patients from five clinical centres.

2. Materials and methods

2.1. Patient and tumour characteristics

DNA-methylation measurements were performed on DNA isolated from snap-frozen primary breast cancers. The unicentre marker discovery study included 39 node-negative and 70 node-positive breast cancer patients (Department of Obstetrics and Gynecology, Technical University of Munich, Germany), who underwent surgery in 1998 or earlier. In the multicentre validation study, 236 independent node-negative breast cancer patients were analysed (Departments of Obstetrics and Gynecology, Technical University of Munich and University Hospital Eppendorf, Hamburg, Germany; Stiftung Tumorbank Basel, Switzerland; Clinical Experimental Oncology Laboratory, National Cancer Institute, Bari, Italy; and Laboratoire d'Oncogénétique, Centre René Huguenin, St. Cloud, France). Inclusion criteria were availability of frozen tumour specimens, T1–3, oestrogen receptor (ER) and/or progesterone receptor (PR) expression, no lymph node involvement (validation phase only), age > 35 years at diagnosis, surgery by 1998, adjuvant tamoxifen monotherapy (indicated duration 5 years), availability of follow-up data and written informed patient consent. Clinical patient characteristics are summarised in Table 1. Note that due to the lack of material (in most cases, only cellular nuclei available), tumour grade and oestrogen receptor protein levels could not be confirmed centrally, but data from medical records of each individual centre were used. Furthermore, tumour cell content could not be determined. However, since the cellular nuclei originated from specimens used for clinical determination of oestrogen receptor protein levels, the specimens were considered appropriate for the reported studies. Median follow-up in patients still alive at the time of analysis was 5.5 years for both studies. Follow-up data were obtained regularly according to local guidelines. Ethical approval for the study was obtained from local ethics committees of each participating centre.

2.2. Determination of ER and PR expression

All tumours were ER and/or PgR positive (either ≥ 10 fmol/mg of cytosolic protein by enzyme immunoassay (EIA) or dextrane charcoal assay (DCC), or immunohistochemical Remmele Score > 0 on a scale ranging from 0 to 12).

Table 1 – Patient characteristics of marker discovery and validation study

	Marker discovery study	Validation study
Number	109	236
Grade		
G1	5	38
G2	60	109
G3	42	82
G4	0	1
Unknown	2	6
Tumour stage		
T1	28	83
T2	54	139
T3	10	6
T4	17	5
Unknown	0	3
Nodal status		
N0	39	236
N+	70	0
Age at diagnosis		
40 and younger	0	3
41–50	7	15
51–60	29	75
61–70	39	79
71–80	34	56
81 and older	3	8
Menopausal status		
Post	105	200
Pre	4	26
Unknown	0	10

2.3. DNA extraction for methylation profiling

Genomic DNA was extracted (QIAamp Blood Kit, QIAGEN, Hilden, Germany) either from deep-frozen 100,000*g* pellets containing cellular nuclei obtained after tumour tissue cytosol preparation, or from snap-frozen tumour tissue.

2.4. Bisulphite treatment, polymerase chain reaction (PCR) amplification, and microarray hybridisation

Bisulphite treatment, PCR amplification and microarray hybridisation were performed as previously described.[7,14] Briefly, regulatory regions of candidate genes were amplified from bisulfite-treated genomic DNA in multiplex PCR reactions labeled with fluorochrome Cy5. Primers were designed ensuring unbiased amplification of methylated and unmethylated alleles. PCR products were hybridezed to microarrays, carrying pairs of immobilized oligonucleotides reflecting the methylated (CG) and non-methylated (TG) status for each position of 1–3 CpG dinucleotides. Microarrays included 499 probe pairs to 117 candidate genes in the marker discovery study and 151 probe pairs representing 33 genes in the validation study. Fluorescent images of the hybridized microarrays were obtained using a GenePix 4000 microarrys scanner (Axon Instruments, Union. City, CA). Median spot intensities of detection oligonucleotides were taken to calculate the methylation score (log [CG/TG]). The methylation score for further analyses was calculated as median over four spot repetitions per chip and over on average four hybridization repetitions (for the gene list, see Supplementary material).

2.5. Quantitative DNA-methylation analysis by real-time PCR (QM-PCR)

Bisulphite-converted DNA from all node-negative patients (of both studies) with sufficient remaining DNA ($n = 245$) was analysed for PITX2 methylation by real-time PCR. The assay format has been described previously.[14] Briefly, in order to ensure unbiased amplification of both methylated and unmethylated states, primers were designed to exclude any CpG dinucleotides. TaqMan probes, specific for methylated and unmethylated states, respectively, were designed to probe the same sequence as covered by the DNA microarray in a duplex probe assay. Threshold cycles obtained on 7900 real-time PCR System (Applied Biosystems) from both probes were used to calculate the methylation score as follows:

$$\text{methylation score} = 100/[1 + 2\{Ctm - Ctu\}],$$

where Ctm and Ctu denote threshold cycles of the probe representing methylated and unmethylated state, respectively.

2.6. Statistical analyses

DNA methylation scores were measured for each probe pair – either from microarray or real-time PCR. The relation between metastasis-free survival times (MFS) and the standardised methylation score of the CpG sites from the microarrays was analysed by linear univariate Cox Proportional Hazard Models.[15,16] Likelihood ratio (LR) tests were performed to test for a significant impact of methylation score at a CpG site on metastasis-free survival. Survival curves were depicted according to the Kaplan–Meier method.[17] Log-rank tests were performed to test for differences between dichotomised survival curves. Multivariable regression analysis was performed by linear Cox Proportional Hazard Models. The models included one of the clinical factors (nodal status (discovery study only), tumour stage, nuclear grade and standardised age at time of diagnosis) and the methylation score of a particular CpG site. Wald tests were performed to test the hypothesis that the CpG site in question provides significant information to the model.

For the technical validation study, the Pearson correlation coefficient between transformed microarray (methylation score: (100*CG/(CG+TG)) and QM-PCR assay data was calculated.

3. Results

3.1. Marker discovery study

Using a methylation microarray, we assessed DNA-methylation scores of 117 different candidate genes for association with metastasis-free survival. The marker discovery study set consisted of 109 primary tumour tissues from consecutive cases of breast cancer patients treated at one clinical centre (Technical University of Munich, Germany). All patients had hormone receptor-positive tumours and received tamoxifen as the only adjuvant therapy. Postmenopausal patients were

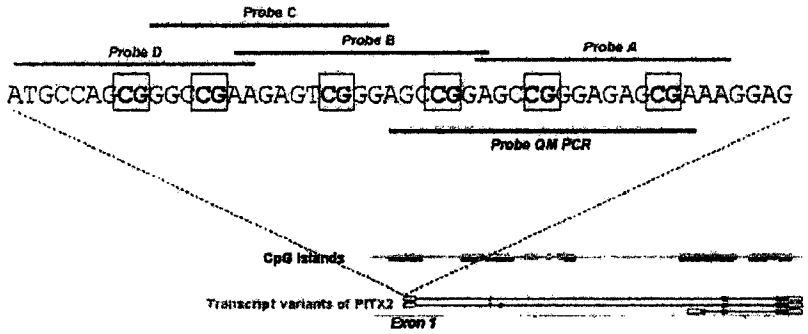

Fig. 1 – Analysed region of the PITX2 gene with details of sequence and detection probes for microarray (A–D, orange) and Quantitative DNA-methylation analysis by real-time PCR (QM-PCR) (blue), respectively. Organisation of PITX2 gene and cytosine phosphoguanine dinucleotides (CpG) Islands (according to NCBI, http://www.ncbi.nlm.nih.gov) is shown at the bottom of the figure.

somewhat over-represented in our data set, most likely due to the fact that premenopausal patients were preferentially treated by chemotherapy at the time our patients had been diagnosed. For this exploratory first step, we included breast cancer patients irrespective of their nodal status (N0: 39; N+: 70). Since nodal status is a strong prognostic factor in breast cancer, lymph node involvement was included as multivariate factor when associating the single CpG sites with survival. Among all candidate genes, DNA methylation of the PITX2 gene showed the strongest correlation to metastasis-free survival. For probe A (covering 2 CpG dinucleotides in the regulatory region of PITX2, Fig. 1), hypermethylation was significantly associated with poor outcome, with a hazard ratio of 2.1 ($p = 0.0014$, Table 2A). For probes B and C, covering CpG dinucleotides in the vicinity, similar results were obtained (Table 2A).

3.2. *Validation study*

To validate our findings, we determined methylation scores of PITX2 in an independent cohort of 236 patients from five clinical centres, using the same microarray technology. Only PITX2 DNA methylation was a significant predictor of outcome in the marker discovery study. However, since potential markers might have been missed in the discovery study due to the low power, we included 32 additional candidates from the discovery step, who had shown a trend for correlation, in the validation study. These additional genes were selected based on the following criteria: first, the ranking of the single CpG sites in the discovery step, second, the degree of co-methylation of several CpG sites within the gene or promoter. Furthermore, we included those genes which had previously been identified as best outcome predictors in advanced breast cancer.[14] Again, all patients had hormone receptor-positive breast cancer and had received tamoxifen as the only systemic adjuvant therapy. Since over-treatment is clinically most relevant in node-negative breast cancer patients, we only included breast cancer patients without nodal involvement in the validation step.

The validation study confirms the significant impact of PITX2 methylation on metastasis-free survival. The CpG site of PITX2 (covered by probe A), which had been identified during the marker discovery step as the best marker for metastasis-free survival, again showed the strongest impact on outcome (univariate Cox regression analysis: hazard ratio = 2.5, $p = 0.000033$, Table 2B). Confirming our previous results, hypermethylation of this site was associated with

Table 2B - Metastasis-free survival: validation study, univariate analysis

	p-Value	Hazard ratio (95% confidence interval (CI))
CpG site A of PITX2	0.000033	2.5 (1.5, 4.1)
CpG site B of PITX2	0.00045	2.1 (1.3, 3.3)
CpG site C of PITX2	0.000021	2.3 (1.4, 3.8)
CpG site D of PITX2	0.11	1.3 (0.9, 1.9)
Tumour stage	0.72	1.1 (0.6, 2.3)
Tumour grade	0.83	0.9 (0.5, 1.9)
Age at diagnosis	0.27	1.2 (0.9, 1.8)

Table 2A - Metastasis-free survival: marker discovery study

CpG sites of PITX2	p-Values of LR-test for complete model	p-Values for CpG sites of PITX2	Hazard ratios for CpG sites of PITX2	p-Values for N-stage	Hazard ratios for N stage
A	0.000036	0.0014	2.1 (1.3, 3.4)	0.0044	5.8 (1.7, 20.0)
B	0.00013	0.0069	2.0 (1.2, 3.3)	0.0057	5.5 (1.6, 18.0)
C	0.00039	0.016	1.8 (1.1, 3.0)	0.0059	5.5 (1.6, 19.0)
D	0.011	1	1.0 (0.6, 1.6)	0.013	4.6 (1.4, 15.0)

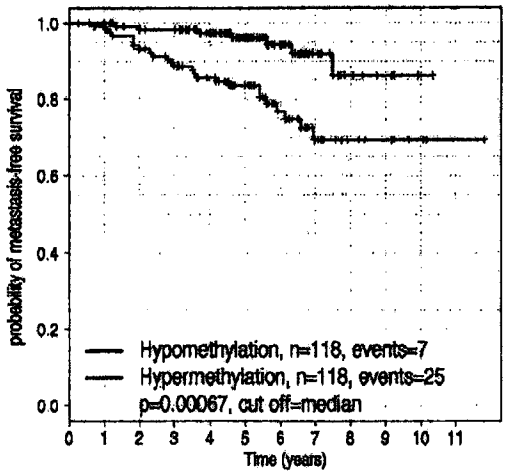

Fig. 2 – Kaplan–Meier curves for metastasis-free survival stratified by DNA-methylation scores of the PITX2 gene (Probe A) in validation study ($n = 236$; N0 patients only). Patients with PITX2 methylation score above median (*hyper*methylation) are depicted in the red (lower) curve; patients with methylation score below median (*hypo*methylation) are indicated in the black (upper) curve. *p*-Value according to Logrank-test.

Table 2C – Metastasis-free survival: validation study, multivariate analysis

	p-Value CpG site	Hazard ratio CpG site (95% CI)	*p*-Value clinical factor	Hazard ratio clinical factor (95% CI)
Tumour stage				
A	0.00024	2.5 (1.5, 4.2)	0.71	1.1 (0.6, 2.4)
B	0.0017	2.1 (1.3, 3.3)	0.72	1.1 (0.6, 2.4)
C	0.0012	2.3 (1.4, 3.8)	0.69	1.2 (0.6, 2.4)
D	0.1	1.3 (0.9, 1.9)	0.65	1.2 (0.6, 2.4)
Tumour grade				
A	0.00024	2.6 (1.5, 4.2)	0.76	1.1 (0.5, 2.3)
B	0.0017	2.1 (1.3, 3.3)	0.86	1.1 (0.5, 2.2)
C	0.0012	2.3 (1.4, 3.8)	0.87	1.1 (0.5, 2.2)
D	0.11	1.3 (0.9, 1.9)	0.99	1.0 (0.5, 2.0)
Age at diagnosis				
A	0.00029	2.5 (1.5, 4.1)	0.41	1.2 (0.8, 1.7)
B	0.0021	2.0 (1.2, 3.2)	0.45	1.1 (0.8, 1.6)
C	0.0015	2.2 (1.4, 3.7)	0.46	1.1 (1.0, 1.6)
D	0.12	1.3 (0.9, 1.9)	0.31	1.2 (0.8, 1.7)

poor metastasis-free survival. Using the median methylation ratio at this site as a cut-off, we defined a low-risk group in which 86% of the patients were metastasis-free at 10 years after surgery compared to only 69% in the high-risk group (Fig. 2). In each of several multivariate models of MFS, including tumour size, grade, or patient age, PITX2 methylation contributed significantly to risk prediction (Table 2C). We also observed a trend towards association of higher PITX2 methylation scores with poor overall survival, although the finding was not statistically significant. In the group with low PITX2 methylation (probe A), 81% of patients were alive at 10 years, compared to 68% in the group with high PITX2 methylation (data not shown). Similar results were obtained for probes B and C.

3.3. *Technical validation of PITX2 by independent DNA-methylation detection technology*

To confirm the PITX2 DNA-methylation scores obtained by microarray, we re-analysed all node-negative tumour specimens from both studies with sufficient remaining DNA ($n = 245$), using a newly developed real-time PCR-based, quantitative DNA-methylation assay (QM assay), covering the same region of PITX2 as the microarray (Fig. 1). DNA-methylation scores calculated from the QM assay were compared with those obtained from the microarray analyses. These PITX2 methylation scores correlated well with those obtained by the QM assay (Pearson correlation coefficient = 0.87, Fig. 3a). Kaplan–Meier curves calculated from the QM assay data provided clinical information comparable to the curves calculated based on the microarray data (Fig. 3b). Thus, the significant correlation of PITX2 DNA-methylation score with outcome is confirmed by an independent method.

4. Discussion

This paper for the first time gives strong evidence in larger patient cohorts that DNA methylation provides clinically relevant information with regard to patient outcome in primary breast cancer. PITX2 methylation was identified as a strong marker for assessment of distant recurrence risk in a cohort of tamoxifen-treated patients, and then successfully clinically validated in a larger, independent cohort. In addition, technical validation was obtained using an independent methodology; moreover, this independent methodology produces entirely consistent clinical implications. Thus, PITX2 constitutes the first independently validated DNA-methylation marker for outcome prediction in primary breast cancer. The results suggest that PITX2 tumour methylation is a clinically useful marker to identify node-negative, hormone receptor-positive patients who are sufficiently treated by adjuvant tamoxifen alone and thus suited to guide physicians and patients in their decision for or against adjuvant chemotherapy in addition to adjuvant tamoxifen. Moreover, these low-risk patients may derive sufficient benefit from adjuvant tamoxifen and – if postmenopausal – may not require *up-front* aromatase inhibitor therapy.

The role of the PITX2 protein in breast carcinogenesis and progression is unclear. We included PITX2 in our list of candidate genes based on a report that described PITX2 methylation in acute myeloid leukaemia.[18] PITX2 encodes a homeodomain transcription factor and is regulated by the WNT/DVL/beta-catenin and hedgehog/TGFβ pathways.[19] The protein is required for morphogenesis of anterior structures such as eyes, teeth and anterior pituitary; it plays a role in left/right patterning[20,21] and is differentially expressed in pituitary adenomas.[20,22,23] Germ-line mutations cause Rieger's syndrome, which is characterised by eye and teeth

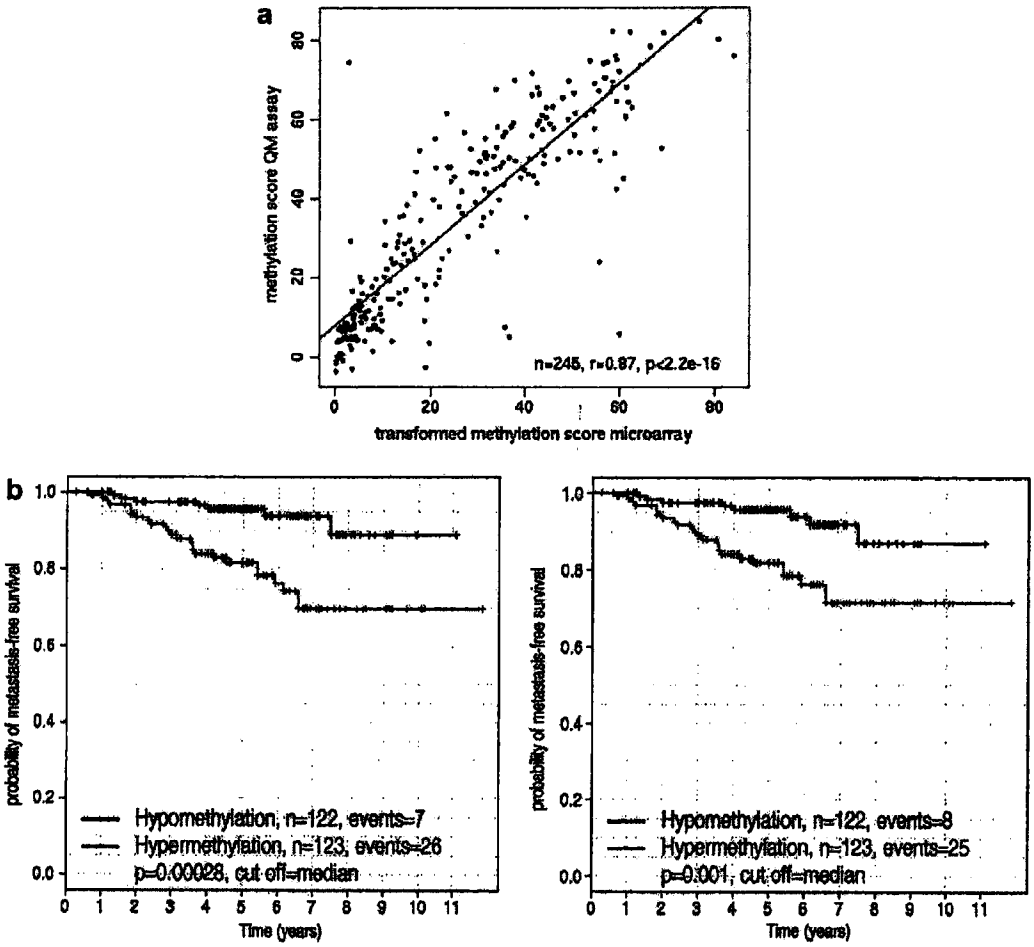

Fig. 3 – Technical (a) and clinical (b) consistency of PITX2 methylation determination by microarray and quantitative methylation analysis by real-time PCR (QM assay). (a) Correlation of DNA methylation scores of the most significant capture probe A of microarray analysis (x-axis) versus QM-assay (y-axis) in 245 node-negative breast cancer patients. (b) Kaplan–Meier curves for metastasis-free survival stratified by DNA-methylation scores of the PITX2 gene, as determined by two different technologies (245 node-negative breast cancer patients from marker discovery and validation study). (Left) Data derived from microarray analysis. (Right) Data derived QM-analysis. Patients with PITX2 methylation scores above median (*hyper*methylation) are depicted in the red (lower) curve; patients with methylation scores below median (*hypo*methylation) are indicated in the black (upper) curve. *p*-Values are according to the Logrank-test.

malformations.[24,25] Interestingly, no association with neoplastic disorders has been reported for Rieger's syndrome. Collectively, these data imply a growth or differentiation control function for PITX2, which could contribute to malignancy when out of equilibrium.

Our results are consistent with an association of PITX2 methylation either with tumour aggressiveness or with tamoxifen response, both of which could contribute to clinical outcome in breast cancer patients treated with adjuvant tamoxifen. In rats, Schausi et al. demonstrated that PITX2 activates the promoter of the truncated ER product-1 (TERP-1),[26] an ER variant which has been shown to be specifically expressed in lactotropic cells of the pituitary gland, thus suggesting that a possible link between PITX2 methylation and endocrine responsiveness. On the other hand, we found that PITX2 methylation was not associated with clinical benefit from first-line tamoxifen monotherapy in endocrine-naïve, metastatic breast cancer,[14] suggesting it may primarily be a marker of tumour aggressiveness and treatment in the adjuvant setting is still early enough to counteract this particular tumour biology whereas treatment in the metastatic setting may be too late. Preliminary results from tumours of untreated patients do suggest that the PITX2 methylation marker also has a prognostic component.[35] These studies will also further explore the relation of PITX2 with other established clinical factors; in the present study, we found that

PITX2 predicts outcome independent of tumour stage, grade and patient age.

The relationship between PITX2 methylation and mRNA expression is unknown. The PITX2 gene generates three major transcripts. Transcripts A and B use one common promoter, whereas transcript C is regulated via an alternative downstream promoter. In our study, we investigated a CpG Island in exon 1, i.e. in the regulatory region of transcripts A and B. Following current assumptions[27] DNA methylation in this area is expected to lead to loss of expression of PITX2 isoforms A and B. Studies testing this hypothesis are currently ongoing and will help to clarify the biological role of PITX2 methylation in breast cancer. Yet, such a deeper understanding is helpful but not a prerequisite for the clinical use of PITX2 as a marker for outcome prediction.

Recently, in endocrine-naïve, metastatic breast cancer, we identified several genes whose methylation score was associated with clinical benefit from first-line tamoxifen monotherapy.[14] Interestingly, none of the genes associated with response in *advanced* breast cancer were a good outcome predictor in the *adjuvant* setting, possibly due to differences in the underlying tumour biology of early and advanced breast cancer, or because markers with a (additional) prognostic component may prevail in the current (adjuvant) study.

Widschwendter et al.[15] described an association of ESR1 hypermethylation with favourable outcome in patients receiving adjuvant tamoxifen. Since high ER levels predict response to tamoxifen,[28] and since ESR1 gene expression is negatively regulated by DNA methylation, [29–31] this finding is quite unexpected. We analysed ESR1 methylation in the same genomic region as Widschwendter et al. in both our discovery and validation study, but did not find a correlation to patient outcome (data not shown). However, in contrast to our study, some of the patients analysed by Widschwendter et al. received additional therapy (i.e. chemotherapy) besides endocrine treatment, complicating the interpretation of their results. In our study, only ER- and/or PR-expressing tumours were included; thus ER expression and consequently DNA methylation of ESR1 is not expected to be a major outcome predictor.

Several groups have identified mRNA expression patterns associated with outcome in breast cancer.[32–34] Van't Veer et al.[32] and Wang et al.[33] identified prognostic signatures in patients without adjuvant systemic therapy. Paik et al.[34] developed a recurrence score for tamoxifen-treated patients based on 16 genes and 5 controls. The score assigns 51% of patients to a low-risk group (10-year-MFS 93.2%), 22% of patients to an intermediate-risk group (10-year-MFS 85.7%), and 27% to a high-risk group (10-year-MFS 69.5%). The performance of their recurrence score and our single methylation marker seems comparable, although caution has to be applied when comparing clinical performance of markers measured in cohorts that are not completely identical (e.g. the 10-year-MFS of our whole cohort was 77.5% versus 85.2% in the study of Paik et al.[34]). Furthermore, the recurrence score of Paik et al.[34] was optimised and grouped into three categories prior to validation, whereas we used a median cut-off to assign patients into two risk groups to avoid potential over-fitting at an early phase in marker development. For eventual routine clinical application, measurement of a single, DNA-based marker may be more practical than a larger, mRNA-based panel. Although the data indicate that mRNA quantification from paraffin-embedded tissue is feasible,[34] DNA-based markers are generally expected to be more robust in a clinical environment.

5. Conclusions

We have described the identification and independent clinical and technical validation of a DNA-methylation marker, PITX2, for outcome prediction in primary breast cancer. The results provide strong evidence that, based on PITX2 methylation alone, up to half of the patients with hormone receptor-positive, node-negative breast cancer can be considered low-risk for distant recurrence if treated by adjuvant tamoxifen and thus may be candidates for being spared adjuvant chemotherapy. Moreover, PITX2 methylation may help to determine a subgroup of postmenopausal patients who do sufficiently well on tamoxifen as their endocrine therapy and may not require up-front aromatase inhibitors. These promising results warrant further studies to determine a clinically optimal cut-off and to address the performance of the marker in larger cohorts in order to allow more detailed subgroup analyses, e.g. in premenopausal patients.

Conflict of interest statement

SM, IN, TK, AK, IS, AM, and RL were employed by Epigenomics AG, a company, which is in the business of commercialising diagnostic products. NH and MS received research support from Epigenomics AG. NH and CT received consulting fees and/or research support from Roche Diagnostics. The other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2007.04.025.

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DNA hypermethylation of *PITX2* is a marker of poor prognosis in untreated lymph node-negative hormone receptor-positive breast cancer patients

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Abstract *Background* In this study, we evaluated if *PITX2* DNA methylation is a marker for disease recurrence in lymph node-negative (LNN), steroid hormone receptor-positive (HR+) breast cancer patients. In addition, we studied the association between *PITX2* DNA methylation and *PITX2* gene expression. *Patients and methods PITX2* DNA-methylation was measured in tumor tissue from 412 LNN/HR+ breast cancer patients who had not received any adjuvant systemic treatment. In addition, PITX2 DNA-methylation and mRNA expression was evaluated in 32 breast cancer cell lines. *Results* In univariate Cox regression analysis, DNA-methylation of *PITX2* as a continuous variable was associated with early distant metastasis (HR = 1.71; $P < 0.01$) and poor overall survival (HR = 1.71; $P < 0.01$). In multivariate analysis together with the established prognostic factors age, tumor size and tumor grade, and steroid hormone receptor levels, both associations retained their significance (for MFS, HR = 1.74; $P < 0.01$; for OS, HR = 1.46; $P = 0.02$). In the breast cancer cell lines, PITX2 DNA methylation was inversely association with *PITX2A* and *PITX2B* mRNA expression ($P < 0.01$). *Conclusions* Hypermethylation of *PITX2* is, in cell lines, negatively associated with PITX2 mRNA expression and, in clinical specimens, positively associated with breast cancer disease progression.

Keywords Breast cancer · DNA-methylation · Estrogen receptor · *PITX2* · Prognosis

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Introduction

Over the last years, several different effective treatment options have become available for early breast cancer. To select the most appropriate therapy for an individual patient, biomarkers predicting the aggressiveness of disease as well as the likelihood of response to an available therapy are urgently needed. DNA methylation is a common and early event in cancer, including cancer of the breast. Gain of DNA methylation is observed in regulatory regions of specific genes which in general adversely affects gene expression [1–5]. Next to their biological significance, DNA-methylation tags have diagnostic potential since they are chemically stable, localized in specific regions, can be readily amplified and is attainable from formalin-fixed paraffin-embedded material [6, 7].

Recently, we analyzed the DNA-methylation status of 117 genes in 109 steroid hormone receptor-positive primary tumors, and found that DNA methylation of the upstream transcription start site (P2 start site) of *PITX2* was the strongest predictor of poor outcome in breast cancer patients with steroid hormone receptor-positive tumors who had received adjuvant tamoxifen monotherapy [8]. This finding was subsequently validated in an independent

study involving 236 steroid hormone receptor-positive lymph node-negative patients who had also only been treated with adjuvant tamoxifen [8].

PITX2 is a bicoid-related homeobox transcription factor that is involved in pituitary-specific gene regulation and left-right patterning during embryonal development [9–11]. Mutations in this gene cause Axenfeld-Rieger syndrome [12]. The human *PITX2* gene drives three major transcripts [12, 13] leading to three different proteins, *PITX2*A, B and C (Fig. 1a). Two promoters, designated P1 and P2, are operational [10, 14]. The P2 promoter drives the transcription of two mRNA variants, leading to the A and B protein. *PITX2*A and B differ in the amino acids encoded by exon 3 [13]. P1 drives the third transcript variant encoding the C protein [14]. The WNT pathway controls the P2 promoter which is of relevance for normal pituitary development [10], while TGFβ family members regulate the P1 promoter that controls through *PITX2*C expression proper asymmetric organ development [14, 15].

The role of the *PITX2* proteins in breast carcinogenesis and progression is unclear. Our results suggest that *PITX2* DNA methylation is connected to tumor aggressiveness and/or to tamoxifen resistance, both of which contribute to clinical outcome in breast cancer patients treated with adjuvant tamoxifen [8]. However, earlier work from our group in clinical specimens did not find *PITX2* DNA-methylation to be associated with intrinsic tamoxifen resistance in metastatic breast cancer [16].

The major aim of the current study was to explore whether DNA methylation of *PITX2* is a prognostic marker. In addition, we studied in cell lines whether *PITX2* DNA methylation was associated with silencing of *PITX2* gene expression. To determine the prognostic value of *PITX2* DNA methylation, we analyzed *PITX2* DNA methylation in a large retrospective cohort of patients who had not received any systemic adjuvant treatment and who were followed for a median time of 93 months. *PITX2* DNA-methylation levels were determined in bisulfite-converted genomic DNA from eligible primary breast tumors by a quantitative DNA-methylation assay (QM-PCR). This validated real-time PCR based assay allows simultaneous quantitative measurement of unmethylated and methylated copies of the *PITX2* gene in genomic DNA [8]. The analyzed CpG sites are located in the region of the P2 start site of the *PITX2* gene. These CpG sites were chosen because they were informative predictors of patient outcome in our earlier study, which only included patients with adjuvant endocrine therapy [8]. Since that study focused on patients with steroid hormone receptor-positive disease, we restricted our current study to a comparable cohort of patients.

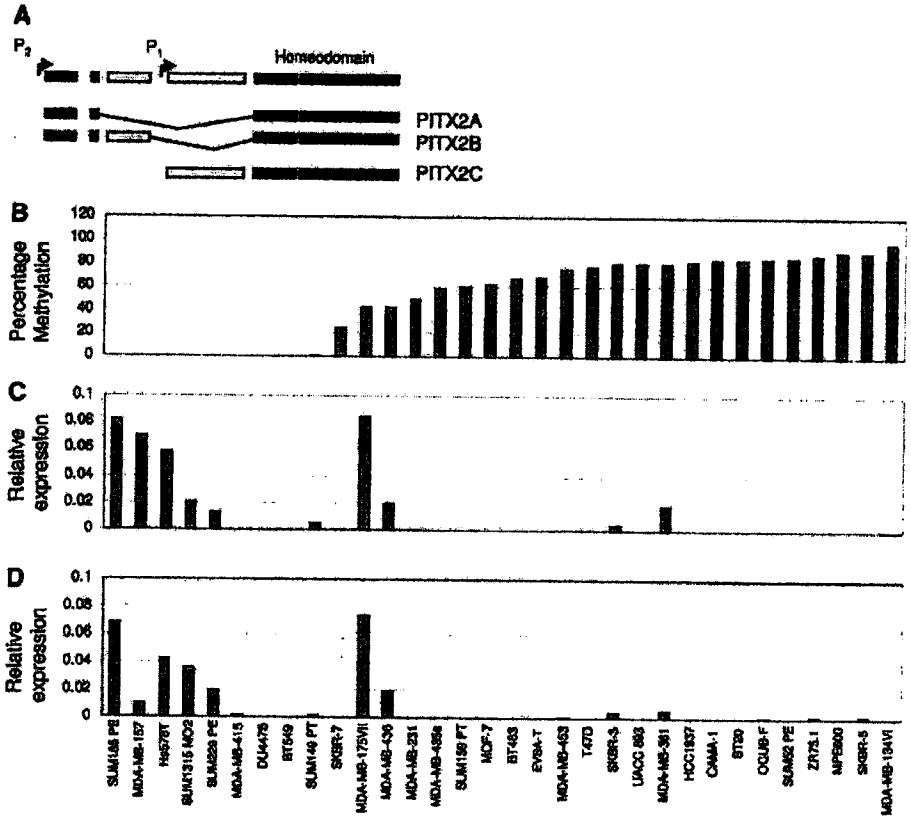

Fig. 1 DNA-methylation of the P2 promoter is inversely associated with PITX2A/B mRNA expression (a) Schematic representation of the *PITX2* gene. The P1 and the P2 promoter, the exon- intron boundaries and the transcribed mRNA species are represented. (b) P2 promoter DNA-methylation determined by bisulfite sequencing. For this, the P2 promoter region was sequenced after bisulfite treatment. DNA methylation status in this graph reflects the percentage of methylated CpG sites in the sequenced region. (c, d) Quantification of *PITX2*A and *PITX2*B mRNA levels. Using real-time PCR, messenger RNA of the *PITX2*A (c) and the *PITX2*B variant (d) were quantified. DNA-methylation and mRNA were expressed per cell line and ranked from left to right by increased methylation status

Patients, materials and methods

Patient and tumor characteristics

A protocol for studying molecular markers associated with disease recurrence was approved by the institution's Medical Ethical Committee (MEC no. 02·953). The present study, in which anonymized tumor tissues were used, was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (http://www.fmwv.nl/). About 415 tumor specimens, obtained at primary surgery between 1982 and 1996, were randomly selected from our liquid nitrogen tumor bank at the Erasmus Medical Center (Rotterdam, The Netherlands). Inclusion criteria were (a) a 100.000×*g* pellet available for DNA extraction; (b) tumors without spread to the lymph nodes at primary diagnosis (negative nodal status); (c) no (neo)adjuvant systemic therapy; (d) tumor size up to 5 cm (T1–T3); and (e) time of surgery in 1996 or before (to allow sufficient follow-up time). *PITX2* DNA-methylation measurement in the primary tumor was successful for 99% ($n = 412$) of the tumors. The median age of the 412 patients at time of surgery was 53 years (range, 36–86 years). Mastectomy was performed on 77 (19%) patients while the remaining patients received breast conserving surgery followed by local radiotherapy. Lymph-node negativity was based on pathological examination that was routinely performed by regional pathologists reflecting routine clinical practice at the time. ER and PgR levels were measured by ligand binding assay or enzyme immunoassay [17] and ≥10 fmol/mg of cytosolic ER and/or PgR protein was considered steroid hormone receptor-positive. All patients were examined routinely every 3–6 months during the first 5 years of follow up and once yearly thereafter. The median follow-up period of patients still alive at time of analysis was 98 months (range, 1–233 months). Of the patients included into the study, 92 (22%) developed distant metastasis during follow-up and were thus counted as events in MFS analysis. Thirteen patients (3%) had died without evidence of disease and were censored at last follow-up in MFS analysis. An additional 87 patients (21%), deceased due to their breast cancer, were counted as events in the analysis for overall survival (OS).

Quantitative DNA-methylation measurement by real-time PCR

Genomic DNA was extracted (QIAamp Blood Kit, QIAGEN, Hilden, Germany) from deep-frozen 100,000×*g* pellets containing cellular nuclei obtained after tumor tissue cytosol preparation [18]. Methylated cytosines in the genomic DNA of tumor tissue specimens were converted to uracils by bisulfite treatment [19] after which the methylation status of the upstream transcription start site (P2 region) of the *PITX2* gene was quantified by real-time PCR (QM-PCR) [8]. The assay uses a standard real-time PCR based technology in which the chosen flanking primers ensure unbiased amplification of the region of interest independent of its methylation status while two fluorescent labeled probes measure the relative methylation levels of 3 GpG sites within the amplified fragment. The reaction mixture (20 µl) consisted of 2 ng bisulfite-converted DNA, PCR amplification primers (100 nM each), FAM- and VIC-labeled probes for specific detection of the methylated and unmethylated variants of the *PITX2* P2 start site region (200 nM each (Applied Biosystems, Darmstadt Germany)), dNTPs, reaction buffer, $MgCl_2$ and Hot-GoldStar-DNA-Polymerase (Eurogentec, Seraing, Belgium). Thermocycling conditions were an initial activation step of 10 min at 95°C, followed by 50 cycles of 95°C for 15 s, and 62°C for 45 s with a single detection of the FAM and VIC signal at the end of the latter step. Cycle threshold values obtained from each probe (Ct_m and Ct_u for methylated and unmethylated status based on the FAM and VIC signal, respectively) were used to calculate the methylation rate ($=100/[1 + 2^{(Ctm-Ctu)}]$).

Methylation status of the P2 regulatory region of the PITX2 gene in breast cancer cell lines

Methylation sensitive restriction. Genomic DNA (4 µg) isolated from breast cancer cell lines was digested with the methylation sensitive enzyme *Hpa*II [20]. To determine if the CpG island of the P2 promoter of the *PITX2* gene was methylated, a PCR (29 cycles and an annealing temperature of 60°C) was performed on digested genomic DNA with primers that flank the P2 regulatory region of the *PITX2* gene (CTCCTCGGTTGGCTCCTAAG and CGTG ACGTCAGCAGAGATTC (253 bp)). Presence of PCR products, analysed on 2.0% metaphore agarose gels, indicated if the region was methylated in one or more of the CpG sites in the P2 region that are part of the *Hpa*II recognition site. Positive controls were included to check for complete digestion as described elsewhere [21, 22].

Bisulfite sequencing. Genomic DNA from breast cancer cell lines was bisulfite treated as described above. The P2 region of the *PITX2* gene was amplified using flanking primers that are specific for successfully bisulfite converted DNA. The retrieved PCR fragment was sequenced using standard procedures to reveal the methylated CpG dinucleotides [23]. The number of CpG sites that were methylated was scored relative to the total number of CpG sites in the sequenced region.

RNA extraction, cDNA synthesis and quantification of PITX2 mRNA species

A detailed procedure for RNA extraction and cDNA sysnthesis from tumor material has been described elsewhere [24]. Briefly, total RNA was extracted with RNABee (Campro, Veenendaal, The Netherlands), quantity and quality checked, reverse-transcribed into cDNA and treated with RNAse H (Ambion, Huntingdon, UK). Real time quantitative PCR on cDNA of the different cell lines was performed using intron-spanning forward and reverse primer combinations (330 nM) and SYBR-green PCR-master-mixture (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) or, for the *PITX2*B variant, Brilliant SYBR Green Master Mix (Stratagene, Amsterdam, The Netherlands). For the *PITX2*A and the *PITX2*B variant the following primer combinations were used (*PITX2*A forward GCGTG TGTGCAATTAGAGAAAG and reverse CCGAAGCC-ATTC TTGCATAG (302 bp); *PITX2*B forward GCCGTTGAATGTCTCTTCTC and reverse CCTTTG CCGCTTCTTCTTAG (198 bp)). Thermocycling conditions were 10 min of activation of the Taq-DNA polymerase at 95°C followed by 35 cycles of 15 s denaturation at 95°C, 30 s annealing at 62°C, 10 s ramping to 72°C, 20 s extension at 72°C, 10 s ramping to 79°C, and 20 s at 79°C. SYBR green fluorescent signals of the products were acquired after each cycle at 79°C. A reference dye, ROX, was included in all assays to normalize data for non-PCR related signal variation. A cDNA pool of human breast fibroblasts and breast cancer cell lines served as positive control and were used for standard curves; negative controls included samples in which reverse transcriptase enzyme was omitted during cDNA synthesis, and samples containing only genomic DNA. Ct values of the gene of interest were used as a quantitative measurement. In cases that *PITX2* transcripts required over 33 rounds of amplification to reach the detection threshold, quantities were considered to be undetectable. *PITX2* levels in these tumors were set to 50% of the expression level measured at the detection threshold. To enable comparison of the levels of specific mRNAs in different samples, they were expressed relative to the average expression levels of three housekeeping genes: porphobilinogen deaminase (PBGD), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and β-2-microglobulin (B2M). The levels of the target genes expressed relative to this housekeeping (HK) set were quantified as follows: mRNA target $=2^{(\text{mean Ct HK} - \text{mean Ct target})}$ [24].

Statistical analyses

To test the association of the level of *PITX2* DNA methylation with continuous variables the Spearman rank correlation test was used while for variables divided over two or multiple groups the nonparametric Wilcoxon rank-sum test and the Kruskal-Wallis test was used, respectively. In cell lines, a χ^2-test was used to associate *PITX2* DNA methylation with *PITX2* mRNA gene expression; in tumors a spearman rank correlation test was used. Uni- and multi-variate Cox regression analysis was used to determine the association of the DNA methylation levels of *PITX2* determined by QM-PCR and/or established clinical-pathological factors such as age, tumor size, and grade with MFS or OS. Hyper- and hypo-methylated groups were defined as indicated. The Cox proportional hazards model was used to calculate the hazard ratios (HR) and their 95% confidence intervals (CI) of covariates in the analyses of MFS or OS. Likelihood ratio test was performed to test whether DNA-methylation or other covariates were related to the hazard. Survival curves were constructed from MFS data using the Kaplan-Meier estimator for survival [25]. Log-rank tests were used to test for differences between two survival curves. All *P*-values are two-sided. Computations were done with the STATA statistical package, release 9.1 (STATA Corp., College Station, TX).

Results

PITX2 DNA-methylation is negatively associated with PITX2 mRNA expression

To determine if *PITX2* DNA methylation is associated with gene silencing, we compared, in a panel of 32 human breast cancer cell lines, *PITX2* DNA methylation and *PITX2* mRNA expression. DNA-methylation of the P2 region was measured in the panel of cell lines with bisulfite sequencing (Fig. 1b) and for conformation by methylation sensitive restriction enzyme digestion (not shown). The results were concordant, revealing that 23 (72%) of the 32 cell lines showed *PITX2* methylation in the P2 regulatory region. Real-time RT-PCR assays specific for the A and the B transcripts of the *PITX2* gene were developed to determine the *PITX2*A and *PITX2*B mRNA expression levels. The specificity of the assays for a particular variant was confirmed in a *PITX2* deficient cell line, CAMA-1, in which the *PITX2*A or *PITX2*B cDNAs were artificially introduced (not shown). The results of the *PITX2*A and B mRNA quantification are shown in Fig. 1c, d, respectively. Eleven of the 32 (34%) of the breast cancer cell lines expressed *PITX2*A and 13 (40%) showed *PITX2*B expression (as cut-off 0.001 was chosen). Almost all cell lines that expressed the A transcript also expressed the B form of *PITX2* which is in line with the view that both transcripts are transcribed from the same promoter. In agreement with the view that DNA methylation silences gene expression, we find a

Table 1 Patient characteristics and their relationship with PITX2 DNA-methylation

Characteristics	No. patients (%)	Median levels (interquartile range)	*P*-value
All patients	412 (100)	21.0 (38.4)	
Age (years)			0.02[a]
≤40	33 (8)	24.1 (38.9)	
41–55	205 (49.8)	17.0 (34.3)	
56–70	125 (30.3)	21.9 (39.6)	
≥71	49 (11.9)	34.1 (43.2)	
Menopausal status			0.02[b]
Premenopausal	207 (50.2)	17.2 (33.6)	
Postmenopausal	205 (49.8)	24.2 (44.9)	
Tumor size			<0.01[b]
T1	269 (65.3)	14.4 (31.9)	
T2/T3	143 (34.7)	30.8 (44.0)	
Tumor grade[c]			0.87[b]
Good/moderate	89 (21.5)	25.2 (32.2)	
Poor	192 (46.6)	17.9 (40.3)	
Unknown	131 (31.9)	21.1 (37.9)	

[a] Spearman rank correlation test

[b] Two-sample Wilcoxon rank-sum (Mann-Whitney) test

[c] Good/moderate tumor grade was compared to poor grade

significant inverse association between *PITX2* DNA methylation and *PITX2*A ($P = 0.004$) or *PITX2*B mRNA expression ($P = 0.005$).

PITX2 DNA-methylation and patient/tumour characteristics

The DNA-methylation status of the P2 region of the *PITX2* gene was measured in tumor DNA from 412 hormone receptor-positive primary breast tumors from patients with no metastasis to the lymph nodes, who had not received any systemic adjuvant therapy. The patient and tumor characteristics are listed in Table 1. The median relative methylation level was 21.1 and ranged from 0.006 to 90.7 for the samples included in this study. The comparison of *PITX2* DNA methylation with patient and tumor characteristics (Table 1) showed that *PITX2* DNA-methylation rates were higher in tumors from older and from postmenopausal patients, in larger tumors, and in tumors with higher ER levels ($r_s = 0.19$; $P < 0.001$).

Univariate and multivariate analysis

In univariate Cox regression analysis, tumor DNA methylation level of *PITX2* measured as a log-transformed continuous variable was associated with early development of distant metastases (HR = 1.71; 95% CI 1.26–2.32; $P < 0.01$, Table 2) and with poor OS (HR = 1.71; 1.25–2.33; $P < 0.01$, Table 3). To visualize the clinical performance of this marker by MFS curves, patients were arbitrarily dichotomized into a hyper- and a hypo-methylation group (33% vs. 67% of patients). Using this cut-point, *PITX2* methylation classified two patient groups with a significant difference in MFS (HR = 1.57; 1.28–1.92; $P < 0.01$) and OS (HR = 1.64; 1.33–2.03; $P < 0.01$) (Fig. 2a, b, respectively). Consistent with our previous study where *PITX2* hypermethylation of the same CpG sites was associated with poor outcome after adjuvant tamoxifen therapy, in this cohort of untreated patients, hypermethylation was associated with a shorter MFS. In multivariate analysis, used either as a continuous variable (for MFS, HR = 1.74; 1.26–2.40; $P < 0.01$; Table 2; and for OS HR = 1.46; 1.05–2.01; $P = 0.02$; Table 3) or as dichotomized as described above in a hyper- and a hypo-methylated group (for MFS, HR = 1.63; 1.30–2.02; $P < 0.01$; Table 2; and for OS, HR = 1.53; 1.21–1.92; $P < 0.01$; Table 3), DNA-methylation of *PITX2* was independent of traditional predictors of poor prognosis: age, menopausal status, tumor size and tumor grade, ER and PgR levels.

Discussion

We have previously shown that DNA hypermethylation of *PITX2* is associated with distant relapse in node-negative

Table 2 Univariate and multivariate analysis for metastasis-free survival of established prognostic makers and PITX2 DNA-methylation

	Univariate analysis		Multivariate analysis ($n = 408$)	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)		$P = 0.16$		$P = 0.28$
≤40	1		1	
41–55	0.57 (0.31–1.06)		0.57 (0.30–1.08)	
56–70	0.49 (0.25–0.96)		0.36 (0.13–1.02)	
≥71	0.39 (0.16–0.98)		0.36 (0.10–1.24)	
Menopausal status		$P = 0.22$		$P = 0.96$
Premenopausal	1		1	
Postmenopausal	0.77 (0.51–1.17)		0.98 (0.44–2.16)	
Tumor size		$P < 0.01$		$P = 0.01$
pT1	1		1	
pT2/3	1.75 (1.16–2.64)		1.72 (1.12–2.65)	
Tumor grade		$P < 0.01$		$P < 0.01$
Poor	1		1	
Unknown	0.61 (0.38–0.98)		0.56 (0.34–0.91)	
Good/Moderate	0.36 (0.19–0.69)		0.38 (0.19–0.74)	
ER status				
Log ER	0.95 (0.84–1.08)	$P = 0.47$	1.07 (0.91–1.25)	$P = 0.41$
PgR status				
Log PgR	0.84 (0.75–0.93)	$P < 0.01$	0.84 (0.75–0.94)	$P < 0.01$
Additions to the base model				
PITX2 DNA-methylation[a]				
Log continuous	1.71 (1.26–2.32)	$P < 0.01$	1.74 (1.26–2.40)	$P < 0.01$
In three quantiles		$P < 0.01$		$P < 0.01$
Low	1		1	
Intermediate	0.76 (0.42–1.37)		0.74 (0.40–1.35)	
High	2.15 (1.32–3.50)		2.29 (1.37–3.82)	
High vs. low/intermediate	1.57 (1.28–1.92)	$P < 0.01$	1.63 (1.30–2.02)	$P < 0.01$

[a] *PITX2* DNA methylation levels were separately introduced to the base multivariate model that included the factors age, menopausal status, tumor size, grade, and ER- and PgR- levels as log-transformed continuous variables

breast cancer patients that had been treated with adjuvant endocrine tamoxifen therapy [8]. In another retrospective study [16], we did not find DNA-methylation of *PITX2* of the primary tumor to be associated with tamoxifen response (given as a first-line single endocrine agent) in metastatic breast cancer. Moreover, none of the strongest predictive DNA-methylation markers of tamoxifen response identified in that study (i.e. *PSAT1, STMN1, S100A2, SYK* and, *PRKCD*) in advanced breast cancer was of significance in predicting disease recurrence in patients treated by adjuvant tamoxifen treated [16].

This could be due to differences in tumor biology between early stage and advanced breast cancer. In this respect, it was already previously discussed that a prognostic factor is not necessarily also a predictive marker, or vice versa [26]. On the other hand, markers with an additional prognostic component may prevail as predictors of disease recurrence in adjuvant-treated patients, since good outcome after adjuvant tamoxifen in these patients may also reflect tumor aggressiveness instead of responsiveness to endocrine therapy. Due to this confounding influence of tumor aggressiveness during adjuvant treatment, it was likely to assume that *PITX2* DNA-methylation may have a prognostic component. The current study demonstrates this for lymph node-negative steroid hormone receptor-positive breast cancer patients. Whether *PITX2* also has a predictive component in early stage breast cancer treated by tamoxifen cannot be concluded from the current study nor from our earlier study in metastatic breast cancer. In order to definitely prove a predictive component, ideally *PITX2* DNA methylation would need to be determined in a retrospective tissue collection from a randomized trial in which one arm received tamoxifen while the other arm received no treatment. Interaction studies would then allow to reveal a predictive component of *PITX2* DNA methylation.

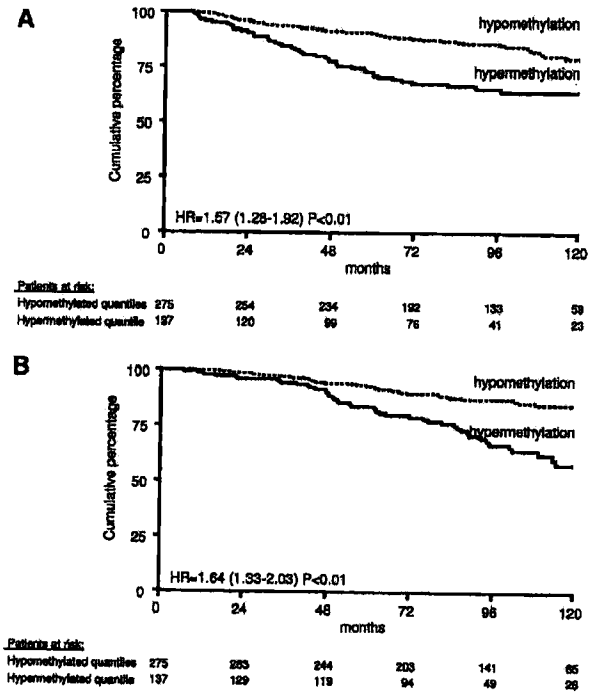

Fig. 2 Kaplan–Meier analysis for MFS dichotomized based on PITX2 DNA-methylation. Kaplan–Meier survival curves for MFS (a) and OS (b) are presented for 412 lymph node-negative steroid hormone receptor-positive patients who did not receive any adjuvant systemic therapy. Patients were divided in three quantiles by the *PITX2* DNA-methylation rate. Due to their comparable hazard, the first two quantiles showing hypomethylation were combined

From a biological point of view, the role of *PITX2* DNA-methylation and in cancer is unknown. Our study shows that hormone receptor-positive breast tumors with a methylated *PITX2* P2 regulatory region are larger, suggesting that these tumors might grow faster although a causal role remains to be demonstrated. In general, DNA-methylation of a regulatory region of a gene is associated with gene silencing due to the recruitment of inhibitory histone remodeling complexes to methylated CpG sites. However, evidence is accumulating that DNA methylation can occur at loci without an effect of gene expression [27]. In addition, complete chromosomal regions can become methylated [27], thus affecting more genes than just the *PITX2* gene alone. The study in the breast cancer cell lines, which we are currently extending to primary tumor specimen, showed an inverse association between *PITX2*A and *PITX2*B mRNA expression and *PITX2* P2 start site DNA-methylation. Thus, altered expression of *PITX2*A and/or *PITX2*B may be underlying the cause of the poor outcome of patients with a methylated *PITX2* P2 region. Presently, the expression pattern of all *PITX2* variants is being analyzed by QPCR in a large collective of primary tumor samples of patients with lymph-node negative disease to get a detailed picture of the tumor biology of *PITX2* expression in breast cancer.

During pituitary development the P2 promoter, that is subject to DNA-methylation, is regulated through a functional TCF binding site under control of the β-catenin pathway [10]. However, whether the P2 promoter is regulated by the WNT pathway in breast cancer, and whether subsequent DNA-methylation of the P2 region interferes with recruitment of the TCF/β-catenin, complex remains to be determined.

A direct role of the estrogen receptor in the regulation of *PITX2* DNA methylation has also been suggested. Not only have estrogen response elements been noted in the *PITX2* gene promoter, it was shown that the *PITX2* gene becomes methylated upon silencing of the estrogen receptor through RNA interference [28]. The increased *PITX2* DNA methylation levels in tumors that are estrogen receptor positive indicate that our in vivo findings are not in accordance with these in vitro observations. *PITX2* has also been associated with the expression of a specific ER splice variant that generates a truncated dominant negative ER protein [29, 30]. Silencing of *PITX2* could thus promote full-length ER signaling and thereby promoting tumor growth of steroid hormone receptor positive breast cancer.

Recently, PITX1, a gene highly homologous to *PITX2*, was identified as a negative regulator of the oncogenic ras pathway. If *PITX2* can perform a similar function, activation of the ras pathway may be underlying poor prognosis in these steroid hormone receptor-positive breast cancer patients.

Concluding, this study shows that DNA-methylation of *PITX2* is an independent prognostic marker for patients with hormone receptor-positive lymph node-negative breast cancer, implying that tumors with a methylated *PITX2* promoter are more aggressive. The fact that in breast cancer cell lines DNA-methylation is inversely associated with *PITX2* mRNA expression motivates biological studies to the role of *PITX2* in tumor growth and metastatic potential of hormone receptor positive breast cancer.

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Conflict of interest statement: Inko Nimmrich, Ina Schwope, Thomas Koenig, Antje Kluth, Oliver Hartmann, Dimo Dietrich, Ralf Lesche, Sabine Maier are or have been employees and/or stockholders of Epigenomics AG (Berlin, Germany), a company that aims to commercialize DNA methylation markers, including PITX2 studied in this manuscript. John Martens, Nadia Harbeck, Manfred Schmitt, and John Foekens are co-inventors of a patent on these DNA methylation markers (WO 2004 035803). This patent has been filed by Epigenomics AG.

Table 3 Univariate and multivariate analysis for overall survival of established prognostic makers and PITX2 DNA-methylation

	Univariate analysis		Multivariate analysis (n = 408)	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)		$P = 0.12$		$P = 0.22$
≤40	1		1	
41–55	0.63 (0.30–1.31)		0.60 (0.28–1.28)	
56–70	0.88 (0.41–1.87)		0.48 (0.15–1.50)	
≥71	1.30 (0.57–2.96)		0.85 (0.25–2.89)	
Menopausal status		$P = 0.07$		$P = 0.51$
Premenopausal	1		1	
Postmenopausel	1.47 (0.96–2.25)		1.34 (0.57–3.15)	
Tumor size		$P < 0.01$		$P = 0.03$
pT1	1		1	
pT2/3	1.88 (1.23–2.86)		1.67 (1.06–2.61)	
Tumor grade		$P = 0.11$		$P = 0.10$
Poor	1		1	
Unknown	0.94 (0.59–1.49)		0.83 (0.51–1.35)	
Good/Moderate	0.54 (0.29–1.01)		0.51 (0.26–0.99)	
ER status				
Log ER	1.09 (0.95–1.25)	$P = 0.19$	1.08 (0.92–1.28)	$P = 0.36$
PgR status				
Log PgR	0.88 (0.79–0.99)	$P = 0.04$	0.88 (0.78–1.00)	$P = 0.04$
Additions to the base model				
PITX2 DNA-methylation[a]				
Log continuous	1.71 (1.25–2.33)	$P < 0.01$	1.46 (1.05–2.01)	$P = 0.02$
In three quantiles		$P < 0.01$		$P < 0.01$
Low	1		1	
Intermediate	0.67 (0.35–1.26)		0.60 (0.31–1.16)	
High	2.25 (1.37–3.70)		1.85 (1.09–3.14)	
High v low/intermediate	1.64 (1.33–2.03)	$P < 0.01$	1.53 (1.21–1.92)	$P < 0.01$

[a] *PITX2* DNA methylation levels were separately introduced to the base multivariate model that included the factors age, menopausal status, tumor size, grade, and ER- and PgR- levels as log-transformed continuous variables

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EXHIBIT D

DNA Methylation Markers Predict Outcome in Node-Positive, Estrogen Receptor-Positive Breast Cancer with Adjuvant Anthracycline-Based Chemotherapy

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Abstract

Purpose: We have shown that DNA methylation of the PITX2 gene predicts risk of distant recurrence in steroid hormone receptor-positive, node-negative breast cancer. Here, we present results from a multicenter study investigating whether PITX2 and other candidate DNA methylation markers predict outcome in node-positive, estrogen receptor-positive, HER-2-negative breast cancer patients who received adjuvant anthracycline-based chemotherapy.

Experimental Design: Using a microarray platform, we analyzed DNA methylation in regulatory regions of PITX2 and 60 additional candidate genes in 241 breast cancer specimens. Using Cox regression analysis, we assessed the predictive power of the individual marker/marker panel candidates. Clinical endpoints were time to distant metastasis, disease-free survival, and overall survival. A nested bootstrap/cross-validation strategy was applied to identify and validate marker panels.

Results: DNA methylation of PITX2 and 14 other genes was correlated with clinical outcome. In multivariate models, each methylation marker added significant information to established clinical factors. A four-marker panel including PITX2, BMP4, FGF4, and C20orf55 was identified that resulted in improvement of outcome prediction compared with PITX2 alone.

Conclusions: This study provides further evidence for the PITX2 biomarker, which has now been successfully confirmed to predict outcome among different breast cancer patient populations. We further identify new DNA methylation biomarkers, three of which can be combined into a panel with PITX2 to increase the outcome prediction performance in our anthracycline-treated primary breast cancer population. Our results show that a well-defined panel of DNA methylation markers enables outcome prediction in lymph node-positive, HER-2-negative breast cancer patients treated with anthracycline-based chemotherapy.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Preliminary results of this study were presented as a poster presentation at 29th San Antonio Breast Cancer Symposium, December 14-17, 2006 [Lesche et al., Breast Cancer Res Treat 2006;100:A6009].

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In breast cancer, currently available methods are inadequate to determine precisely the aggressiveness of the disease and the likelihood of response to a certain treatment in individual patients. Therefore, biomarkers predicting the tumor's metastatic potential and its response to a specific treatment are urgently needed. This is particularly true for patients for whom current guidelines (1) recommend anthracycline-based chemotherapy. These patients comprise a clinically distinct intermediate- to high-risk subgroup, for which in view of the short and long-term toxicity as well as the availability of new effective anthracycline-free chemotherapy options (2), the widespread use of adjuvant anthracyclines is currently under debate (3). Unfortunately, reliable biomarkers for benefit from anthracycline therapy are still lacking.

DNA methylation of CpG dinucleotides within gene regulatory regions, associated with suppression of gene expression, is a common and early event in cancer (4–6). Specific DNA methylation patterns for tumor subtypes including breast cancer have been reported and associated with clinical outcome (7–16).

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Translational Relevance

Outcome prediction of node-positive patients having received anthracycline-based chemotherapy is of clinical relevance particularly in patients with hormone receptor-positive/HER-2-negative disease. Current guidelines recommend anthracycline-based chemotherapy for such patients, but their clinical outcome remains quite diverse. Patients who have a poor outcome may be recommended for currently available anthracycline-free alternative treatment options, whereas patients with a good outcome on anthracyclines are adequately treated. Reliable biomarkers for benefit from anthracycline therapy for these type of patients are, however, lacking. We have identified a panel of DNA methylation markers that may allow reliable prediction of clinical outcome for these patients. Such a panel, when properly validated in independent multicentric cohorts, may be used to recommend anthracycline-based chemotherapy for node-positive breast cancer patients with hormone receptor-positive/HER-2-negative disease.

We have recently found and validated that DNA hypermethylation of the PITX2 promoter is associated with a high risk of recurrence in node-negative, steroid hormone receptor-positive breast cancer who received tamoxifen as their only systemic adjuvant therapy (13, 17). These studies further showed that DNA methylation can be successfully measured in formalin-fixed, paraffin-embedded breast cancer specimens, which is one of the strengths of DNA methylation technologies and considered a prerequisite for routine clinical application (17). A follow-up study revealed that PITX2 methylation is a strong and pure prognostic factor in patients with node-negative, steroid hormone receptor-positive breast cancer who did not receive any adjuvant systemic treatment (15).

In the present study, we have used a microarray technology to study whether methylation of PITX2 and 60 other candidate genes could be predictors for outcome in breast cancer patients with node-positive, steroid hormone receptor-positive, and HER-2-negative tumors receiving adjuvant anthracycline-based chemotherapy. In our study, we focused on patients with lymph node-positive, estrogen receptor (ER)-positive, and HER-2-negative breast cancer, as these patients comprise a clinically distinct subgroup for which anthracycline-based adjuvant chemotherapy is currently recommended (1). Our study suggests that reliable prediction of clinical outcome for these breast cancer patients may be possible using a well-defined panel of DNA methylation markers.

Materials and Methods

Patients and samples

The study cohort comprised 384 node-positive breast cancer patients whose tumor samples were recruited from four clinical centers: Erasmus Medical Center; Centre René Huguenin; Stiftung Tumorbank; and Department of Obstetrics and Gynecology, Technical University of Munich. Appropriate consent, according to institutional requirements, has been obtained for all patients. The study protocol was approved by the local ethics committees.

To be eligible for this study, patients had to fulfill all of the following criteria: (*a*) histologically confirmed invasive breast cancer, (*b*) primary tumor stage pT_1 to pT_3, (*c*) histologically confirmed lymph node involvement (pN > 1), (*d*) surgery before 2002 (potential follow-up of at least 5 years), (*e*) standard adjuvant anthracycline-based chemotherapy [no dose-dense therapy, no other primary systemic chemotherapy (except hormonal therapy), and no additional taxane], and (*f*) availability of clinical follow-up data.

Among the 384 patients recruited for the study, 284 were reported as ER-positive. HER-2 status was determined in all samples using the Light Cycler HER-2/*neu* DNA quantification Kit (Roche Applied Science). HER-2-positive patients (n = 43) who nowadays would receive trastuzumab treatment were then excluded, leaving a total of 241 node-positive, ER-positive, and HER-2-negative breast cancer patients available for analysis. For the whole cohort, the percentages of patients with distant metastasis at 5 and 10 years were estimated as 70.1% (64.3-76.3%) and 56.1% (48.5-65.0%), respectively; 5- and 10-year disease-free survival (DFS) were 64.5% (58.6-71.0%) and 47.4% (40.0-64.2%), respectively; and 5- and 10-year overall survival (OS) were 85.8% (81.4-90.5%) and 67.7% (59.8-79.1%), respectively. The median follow-up 81.5 months.

DNA extraction and bisulfite treatment

Genomic DNA was extracted from snap-frozen tumor tissue or tumor cell nuclei pelleted at 100,000 × *g* as described (14). Bisulfite conversion of the DNA was done using the EpiTect Kit (Qiagen).

Candidate gene selection

The 61 candidate genes (listed in the Supplementary Material) to be analyzed on the microarray were selected based on (*a*) genome-wide screens for prognostic markers in ER-positive and ER-negative breast cancer,[6] (*b*) PITX2 pathway analyses, and (*c*) literature.

PCR amplification and microarray hybridization

PCR amplification and microarray hybridization were done as described previously (7, 14). In total, 64 PCR amplificates representing 61 genes were pooled and hybridized to the microarray on which detection oligonucleotides for methylated (CG) and nonmethylated (TG) gene copies were spotted. This allowed for simultaneous quantitative measurement of unmethylated and methylated copies of the genes. Microarrays included 4 oligonucleotide pairs for each of the 64 PCR amplificates (total of 256 pairs). Each probe pair covered between one and three CpG dinucleotides in the regulatory regions of the respective candidate gene. The methylation score for each CpG site was calculated from the fluorescence intensity values of the methylated (FI_m) and unmethylated (FI_u) oligonucleotides. To stabilize the variance, the score was transformed using the generalized log transformation (gLOG): methylation score = $g\mathrm{LOG}(FI_m / FI_u)$ (18). For statistical analysis, methylation scores for each amplificate were determined by averaging measurements from all probe pairs belonging to one amplificate using the median. Multiple amplificates from the same candidate gene entered data analysis independently. Valid microarray results were obtained for all samples.

Clinical endpoints

The primary clinical endpoint in this study was time to distant metastasis (TDM). Secondary endpoints were DFS and OS. For TDM, only distant recurrences were considered. Ipsilateral and locoregional recurrences were not considered as events or censoring events. Contralateral breast cancer, development of other primary tumors, death (from any cause) without observed recurrence, and loss for follow-up were considered censoring events. For DFS, all recurrences were considered as events, whereas death (from any cause) before recurrence, contralateral breast cancer, and development of other

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primary tumors or loss for follow-up were considered censoring events. For OS, only death (from any cause) was considered as event and patients were censored only when lost for follow-up.

Statistical analyses

The relation between clinical endpoints and DNA methylation score was analyzed for each amplificate by linear univariate Cox proportional hazards models (19, 20). Here, likelihood ratio tests (LRT) were done to test for a significant relationship of methylation scores of each amplificate with clinical endpoints. Hazard ratios (HR) for continuous variables were calculated relative to an increment of the interquartile range of that variable (for the increment from the 25% quantile to the 75% quantile of the measurements; ref. 20). Multivariate regression analysis, testing the association between clinical endpoint and multiple methylation scores and/or clinical variables, was done by linear Cox proportional hazards models. In that case, LRT was done to test for a significant association of the outcome variables (TDM, DFS, and OS) with the derived model consisting of clinical variables and/or methylation scores. In addition, Wald tests (testing the hypothesis that the variable in question provides significant information to the multivariate model) were calculated. Survival curves were plotted according to Kaplan-Meier (21). Log-rank tests were used to test for differences between survival curves. To describe and compare the predictive performance of each variable and each multivariate model, the concordance index (C index; refs. 20, 22) was calculated. To penalize overfitting by entering multiple factors, the bootstrap-corrected version of the C index is given (20, 22).

To correct for multiple testing in univariate testing (64 amplificates), significance levels were adjusted using false discovery rate correction using the linear step-up procedure proposed in ref. 23.

Marker panel feature selection. A nested cross-validation/bootstrap procedure was applied to perform feature selection and panel validation (24, 25). A schematic illustration of the methodology is shown in the Supplementary Material. To identify a marker panel for outcome prediction, the 63 amplificates [all but the amplificate designed in the promoter of transcripts A and B for PITX2 (PITX2P2)] were first ranked by univariate performance (Cox proportional hazards model, LRT). Then, marker panels containing PITX2P2 and increasing numbers of the best 19 single markers were evaluated with respect to their prognostic predictability, which was estimated using the bootstrap-corrected C index with $B = 200$ bootstrap runs (20). A gene was selected for the final predictor model if the C index of the model including this marker was larger than that of the model excluding the marker. A C index was considered "larger" if at least an increase of 1% over the model containing PITX2P2 was observed. The extensive search was limited to a maximum of 20 amplificates altogether, balancing the critical sample size to build a reasonable regression model (number of distant metastases) on the one side and the request to find markers complementing previously included ones (and therefore not necessarily good univariate markers) on the other.

Marker panel validation. The cross-validated prediction score is an unbiased estimate of the performance of the marker panel in a future data set. It corrects for the optimism introduced due to feature selection within the same data set (24, 25). A schematic illustration of the methodology is presented in the Supplementary Material. In brief, the generalization error for the marker panel selection was estimated by repeating the feature selection procedure using a 20-fold cross-validation, starting with all 64 amplificates in each cross-validation run. We trained on 95% of the samples and computed the cross-validated predictive index for the samples in the remaining 5% of samples using the Cox model developed for the training set. To combine results from all runs, the predictive indices for all samples were transformed into risk percentiles within each run. The nested cross-validation procedure itself was replicated 100 times using random permutations of the data set to determine the estimation error of the cross-validated C index. As cross-validated risk percentiles are correlated among cases, the usual null distributions of the test statistics are not valid. To derive P values of both the likelihood ratio and Wald tests (Cox model) as well as the log-rank test (Kaplan-Meier analysis) for the cross-validated prediction score, the null distribution was estimated using 100 repetitions of the entire procedure for permutations of survival times and censoring indicators.

To further illustrate the performance of the cross-validated prediction score, the score was analyzed in a similar way as described above for our individual amplificates. All results using a single cross-validated prediction score were generated using results of the first replicate of the nested cross-validation procedure. Statistical analysis was done using R version 2.4.1[7] and the R package "Design" version 2.1.

Results

Patient and tumor characteristics. Patient and tumor characteristics ($n = 241$) are given in Supplementary Material, and these are comparable with previously published cohorts with similar inclusion criteria (26). These clinical standards may differ somewhat from current guidelines as shown by the fact that only 104 (43%) patients received additional adjuvant endocrine treatment, although their tumors had positive steroid hormone receptor status. All patients had received adjuvant anthracycline-based chemotherapy according to clinical standards at the time of their primary therapy.

Tumor size (HR, 1.77; $P = 0.0225$), grade (HR, 2.07; $P = 0.004$), and adjuvant endocrine therapy (HR, 0.49; $P = 0.0025$) were significantly associated with TDM in this cohort (Table 1). Adjuvant endocrine therapy, grade, and progesterone receptor (PgR) were significantly associated with DFS (data not shown). Tumor size, grade, endocrine therapy, and PgR status were significantly associated with OS (data not shown). For all clinical endpoints, risk of recurrence was lower for patients who received adjuvant endocrine therapy.

Univariate analysis of methylation markers. Because we have observed DNA methylation of PITX2 as a strong marker in previous studies, we first analyzed the performance of this marker. The methylation score measured with the amplificate designed for the promoter of transcripts A and B of PITX2 (PITX2P2), located upstream of the first transcriptional start, showed that PITX2P2 hypermethylation was associated with a high risk of distant recurrence in this patient cohort [HR, 1.66; 95% confidence interval (95% CI), 1.21-2.28; $P = 0.002$; C index = 0.624; see Table 1 and Fig. 1]. PITX2P2 hypermethylation was also associated with poor DFS (HR, 1.47; 95% CI, 1.11-1.96; $P = 0.0084$) and OS (HR, 2.07; 95% CI, 1.40-3.06; $P = 0.0003$). However, methylation of the amplificate designed for the promoter regulating transcript C of PITX2 (PITX2P1) was not associated with distant recurrence in this patient cohort (HR, 1.23; 95% CI, 0.89-1.68; $P = 0.21$).

Among the other 62 tested amplificates, 15 amplificates, derived from 14 genes, were associated with TDM (false discovery rate < 5%; Table 2). Methylation of BMP4, FOXL2, LMX1A, C20orf55, BARX1, PLAU (both amplificates), FGF4, NR5A1, BRCA1, TLX3, NR2E1, LHX4, ZNF1A1, and CCND2 and BMP4, FOXL2, LMX1A, FGF4, and BRCA1 showed C indices between 0.58 and 0.63 comparable with that of PITX2P2. For DFS, BMP4, FOXL2, and C20orf55 and for OS all 15 amplificates described above were significantly associated with the respective clinical endpoint (false discovery rate < 5%;

[7] http://www.r-project.org

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Table 1. Univariate and multivariate Cox proportional hazards analysis for TDM

	Univariate analysis			Multivariate analysis		
	No. samples	HR (95% CI)	P*	No. samples	HR (95% CI)	P*
PITX2P2 methylation †	241	1.66 (1.21-2.28)	0.002‡	200	1.28 (1.03-3.83)	0.026‡
Age at surgery †	241	1.00 (0.75-1.32)	0.981	200	1.01 (0.98-1.04)	0.7
T stage (T_2,T_3 vs T_1)§	239	1.77 (1.08-2.91)	0.023‡	200	1.47 (0.84-2.59)	0.18
PgR status (positive vs negative)§	241	0.77 (0.43-1.36)	0.367	200	1.11 (0.54-2.31)	0.77
Endocrine treatment (yes vs no)§	239	0.49 (0.30-0.78)	0.003‡	200	0.58 (0.33-1.00)	0.052
Grade (1/2 vs 3)§	203	2.07 (1.27-3.83)	0.004‡	200	1.68 (1.00-2.81)	0.049‡

*P values refer to LRT for univariate analysis and Wald test for multivariate analysis.
† PITX2P2 methylation and age at surgery were analyzed as continuous variables (95% CI), and HRs were calculated relative to an increment from the lower quartile to the upper quartile.
‡ Significant feature.
§ T stage, endocrine treatment, PgR status, and grade were analyzed as binary variables.

data not shown). For all markers, with the exception of C20orf55, lower methylation scores were associated with better clinical outcome.

Multivariate analysis of methylation markers and clinical variables. PITX2P2 methylation was a significant marker in the multivariate analysis including age at surgery, pathologic T stage and grade, PgR status, and adjuvant endocrine therapy ($P = 0.05$; Table 1). To show clinical usefulness of a biomarker, it is necessary that the marker increases the predictive performance of established clinical variables. For the multivariate model including PITX2P2, the bootstrap-corrected C index, which is an unbiased estimate of the effect size for regression models, is larger than that for the model including the clinical variables only (0.647 compared with 0.61, respectively; Table 3). Therefore, PITX2P2 provides additional information for prediction of TDM independent of established clinical variables.

Most of the 15 amplificates identified in univariate analysis were also a significant marker in multivariate analysis when combined with age, pathologic T stage, PgR status, and adjuvant endocrine therapy. The bootstrap-corrected C indices of the multivariate models (range, 0.620-0.665) were larger than those of the multivariate model using only clinical variables (0.610; Table 2).

Marker panel selection and performance prediction. Having established the role of several DNA methylation markers (PITX2 and 15 other markers) for outcome prediction, we assessed if combining these markers into a panel resulted in improved performance compared with that of the individual markers alone. Overfitting becomes a critical issue if feature selection, model building, and performance estimation are done within one data set (27). Therefore, the bootstrapped-corrected C index is used in the feature selection step. To select relevant markers that could improve the performance of our already well-established marker PITX2, we evaluated the gain in estimated effect size (C index) by stepwise adding the best markers from univariate analysis to PITX2P2. Figure 2A illustrates the results of the feature selection step. With PITX2P2 as "anchor," BMP4, C20orf55, and FGF4 were selected to define a four-marker panel for prediction of TDM in the current cohort.

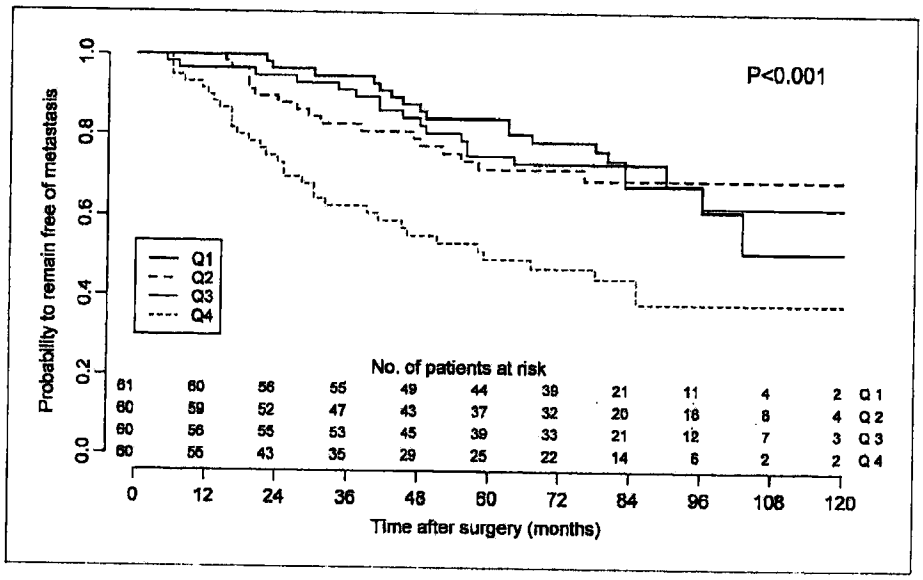

Fig. 1. Prediction of TDM survival probabilities based on PITX2P2 DNA methylation. Kaplan-Meier TDM curves of patients categorized in subgroups based on PITX2P2 DNA methylation scores ($P = 0.00014$). The methylation score was grouped into quartiles. Log-rank test was used to test for significant separation between the groups. C index was 0.624. Patients at risk for the different quartiles are indicated in 12-mo intervals.

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Table 2. DNA methylation markers in ER-positive, HER-2-negative patients that meet the 5% false discovery rate limit for TDM

No.	Gene	Univariate analysis		Multivariate analysis	
		C index	P*	C index†	P*
1	BMP4	0.632	<0.0001	0.680	0.0002
2	FOXL2	0.632	0.0001	0.640	0.02
3	LMX1A	0.617	0.0008	0.635	0.068
4	C20orf55	0.592	0.0009	0.637	0.001
5	BARX1	0.613	0.0015	0.624	0.1
6	PLAU Amp2	0.598	0.0017	0.620	0.12
7	FGF4	0.619	0.0019	0.646	0.029
8	NR5A1	0.609	0.0027	0.635	0.059
9	BRCA1	0.631	0.0028	0.647	0.018
10	TLX3	0.583	0.0032	0.634	0.008
11	NR2E1	0.592	0.0054	0.643	0.02
12	LHX4	0.576	0.0076	0.621	0.12
13	PLAU Amp1	0.585	0.0078	0.635	0.051
14	ZNF1A1	0.594	0.008	0.636	0.044
15	CCND2	0.598	0.01	0.631	0.067

NOTE: For all variables, except C20orf55, low methylation scores were associated with long TDM. Multivariate analyses were done including the respective amplificate as well as age at surgery, T stage, endocrine treatment PgR, and grade.
*P values refer to LRT for univariate analysis and Wald test for multivariate analysis (not corrected for multiple testing).
† C index of multivariate model with clinical variables alone (age at surgery, T stage, PgR, endocrine treatment, and grade) was 0.61.

To estimate the performance regarding outcome prediction of the four-marker panel, feature selection was nested within a cross-validation procedure. This corrects for overfitting due to feature selection, and the resulting cross-validated C index is an unbiased estimate of the performance of the marker panel in a future data set. The C index of the cross-validated prediction score is 0.668 compared with 0.624 for PITX2P2 alone (Table 3). We replicated the estimation procedure 100 times, thereby generating a range of C indices for our four-marker panel. As a result, a median C index of 0.654 (range, 0.622-0.676) was obtained for the prediction score, and in 98 of 100 replications, the estimated performance of our four-marker panel was better than that of PITX2P2 alone (Fig. 2B). Based on these results, we conclude that a four-marker panel can indeed improve outcome prediction over a single marker.

To further illustrate the performance of our outcome predictor, we report the results of the first cross-validation replicate in more detail. The four markers selected for our marker panel represent a stable selection: C20orf55 was selected in all cross-validation runs, FGF4 in 16 of 20 runs, and BMP4 in 14 of 20 runs. Only two other markers were selected in any run: APC and BRCA1 (Fig. 2C). The C index of the cross-validated prediction score for a multivariate model including the cross-validated prediction score as well as age at surgery, pathologic T stage, PgR, and adjuvant endocrine therapy was 0.695 ($P < 0.01$, LRT; Table 3). The Wald P value for the cross-validated prediction score in the multivariate model was <0.01. Kaplan-Meier estimates illustrate the difference ($P < 0.01$, log-rank test; Fig. 2D) between the performance of the identified marker panel (Fig. 3, *left*) and PITX2P2 alone (Fig. 1). For all patients, the 5-year survival prediction of the quartile with the highest cross-validated prediction scores for the four-marker panel is <50%, whereas that of the quartile with the lowest cross-validated prediction scores is >85%. Similar to TDM, significant prediction scores were derived for DFS ($P = 0.01$) and OS ($P < 0.01$). Since today all patients with steroid hormone receptor-positive breast cancer will receive adjuvant endocrine therapy, we performed a subgroup analysis for those patients in our cohort who had received adjuvant endocrine treatment. Although the size of cohort was limited to 104 patients, a highly statistically significant separation of survival groups was observed when stratifying the patients according to the prediction score (Fig. 3, *right*).

Discussion

According to current guidelines, adjuvant chemotherapy needs to be considered for the majority of patients with primary breast cancer, with the exception of a small subgroup with very favorable outcome prediction based on clinical factors (1). In node-positive breast cancer, anthracycline-based adjuvant chemotherapy has become the standard of care during the 1990s (28). Moreover, several studies showed that adding a taxane (29), or, more recently, administering dose-dense chemotherapy regimes (including anthracyclines; ref. 30), further improves outcome in node-positive breast cancer. However, not all patients will benefit from such more aggressive therapies and therefore only face their more severe side effects, because these patients either have an excellent prognosis or have tumors that do not respond to certain chemotherapies. Thus, prognostic and predictive markers are urgently needed to tailor the best possible therapy regimen for the individual patients.

Table 3. Predictive performance estimated from multivariate Cox proportional hazards analysis for TDM

Features in Cox model (TDM)	C index	P (LRT)
Age at surgery, T stage, PgR, endocrine treatment, grade	0.61	0.01
PITX2P2	0.624	0.0019
Age at surgery, T stage, PgR, endocrine treatment, grade, PITX2P2	0.647	0.0029
Four-marker panel	0.668	<0.00001
Age at surgery, T stage, PgR, endocrine treatment, grade, four-marker panel	0.69	<0.00001

NOTE: T stage, endocrine treatment, PgR status, and grade were analyzed as binary variables. PITX2P2 methylation score and age at surgery were continuous variables. For the four-marker panel, the cross-validated prediction score was used as a continuous variable. LRT P values as well as the C index (bootstrap corrected) are calculated for each Cox model.

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In previous studies, we found that DNA methylation of PITX2 reliably predicts the risk of distant recurrence in node-negative breast cancer (13, 15, 17). These results prompted us to analyze PITX2 methylation in node-positive breast cancer and to measure other DNA methylation markers, with the goal to develop a marker panel for improved outcome prediction. For this purpose, we have analyzed a cohort of 241 patients with lymph node-positive, ER-positive, and HER-2-negative breast cancer, who had all received adjuvant anthracycline-based chemotherapy. We focused on patients with these tumor characteristics, because they constitute a clinically relevant subgroup for which standardized treatment guidelines are available (1, 26). Among the node-positive breast cancer patients, our subpopulation is considered to have a relatively favorable outcome because of their positive steroid hormone receptor status and the absence of HER-2 amplification. Therefore, we hypothesized that it might be possible to further stratify this group of patients so that a good prognosis group could be defined that would derive sufficient benefit from conventional anthracycline-based therapy and would not require more aggressive therapy such as, for example, dose-dense regimens or addition of taxanes.

In the current study, we were able to show that PITX2 DNA methylation also predicts outcome in node-positive breast cancer. Because clinical factors such as grade, tumor stage, or age have been described previously as outcome predictors in primary breast cancer, we first calculated a base model. The predictive accuracy of the multivariate Cox model with established clinical variables, quantified using the bootstrap-corrected C index as an unbiased measure of the predictive strength of a model, was 0.604. Adding PITX2 to the base model resulted in a significantly improved outcome prediction (C index = 0.651). This confirms the utility of PITX2 as a marker for outcome prediction in primary breast cancer and

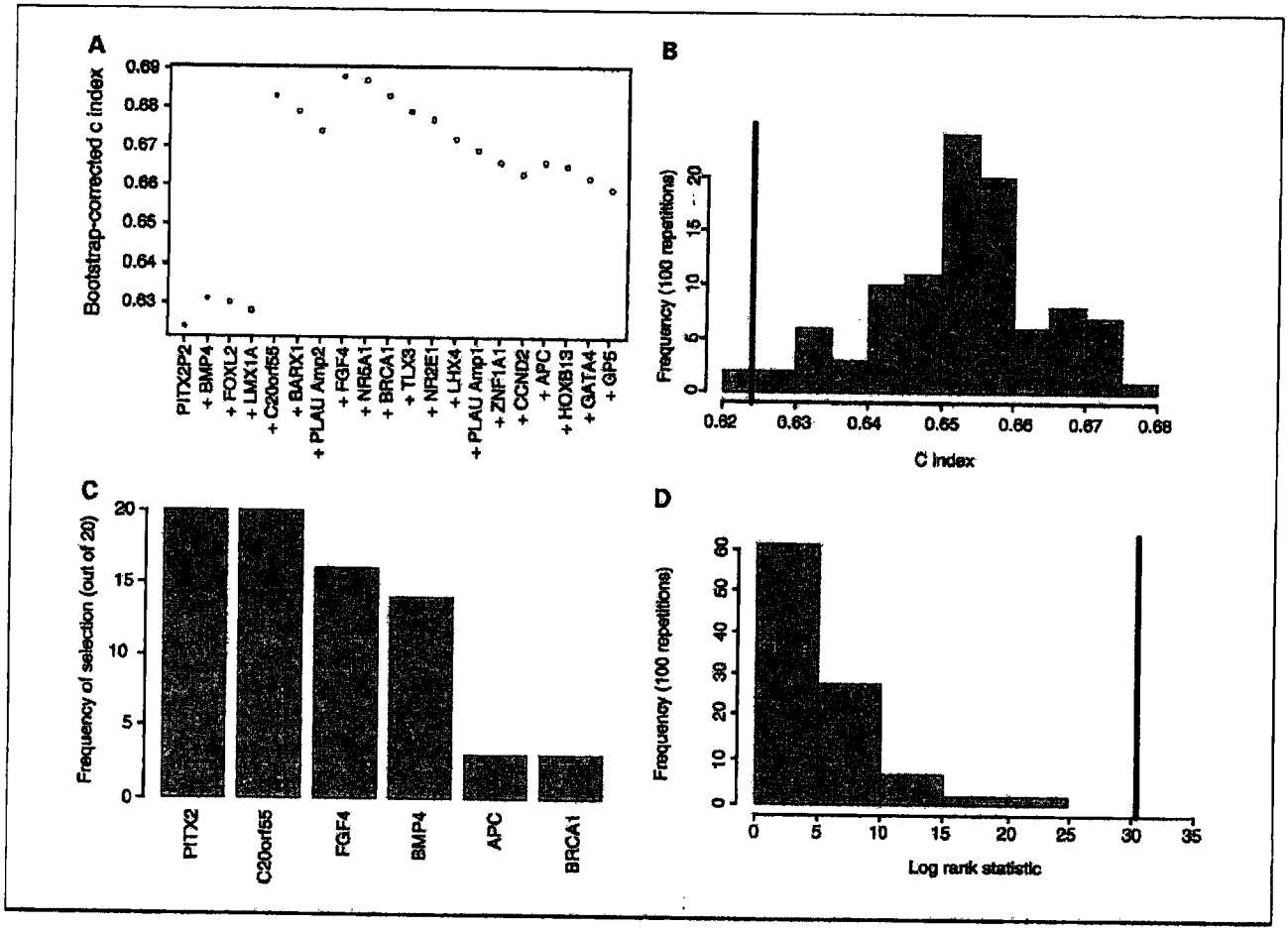

Fig. 2. Marker panel feature selection and marker panel validation. *A*, marker panel selection. Result of the feature selection step using bootstrap-corrected C index to select markers for the panel to predict TDM. Marker models built using PITX2P2 and the first *n* amplificates (*X axis*, markers ranked according to univariate performance) are evaluated using the bootstrap-corrected C index (*Y axis*). *Closed circles*, selected markers. These are, from *left* to *right*, amplificates for PITX2P2, BMP4, C20orf55, and FGF4. *B*, histogram of cross-validated C index from 100 repetitions. C index for model with PITX2P2 alone was 0.624 (*thick vertical black line*); 98 of 100 repetitions for the four-marker panel are larger. *C*, stability of marker selection. Frequency of the indicated markers in cross-validation runs. In each of the 20 cross-validation runs, feature selection is repeated on 95% of the samples. C20orf55 was selected in all cross-validation runs, FGF4 in 16 of 20 runs, and BMP4 in 14 of 20 runs. Only two other markers were selected by this procedure: APC and BRCA1, which were both selected in only 3 of 20 runs. *D*, histogram of the null distribution of the log-rank test statistic of our data set. Histogram shows the distribution of log-rank test statistic of 100 data sets, of which survival time and censoring indicator were randomly permutated. This permutated data set was used to derive *P* values of the LRT (Cox model) for the cross-validated prediction score. *Thick vertical black line*, log-rank statistic for the cross-validated prediction score of the Kaplan-Meier TDM curves shown in Fig. 3 (*left panel*).

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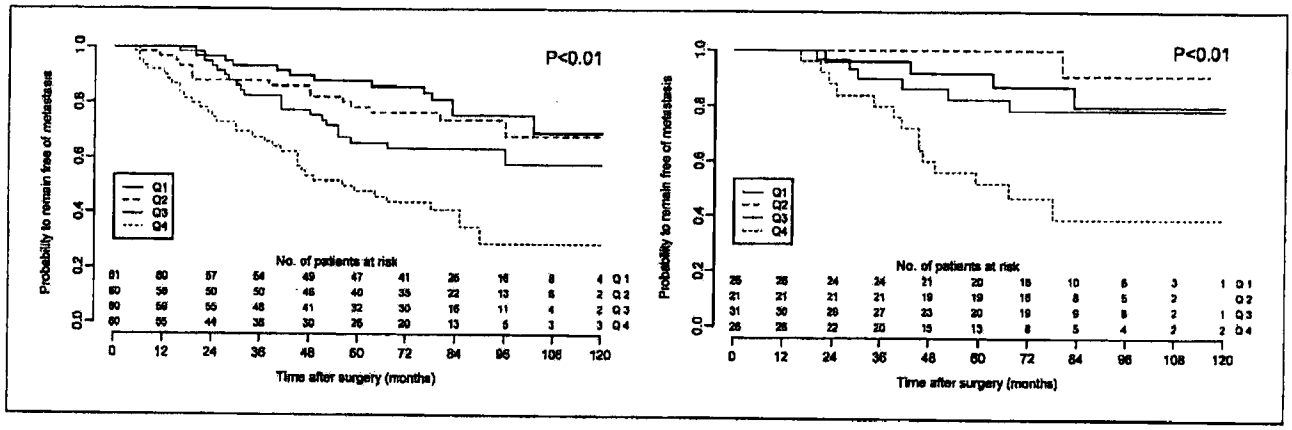

Fig. 3. Prediction of TDM survival probabilities based on the four-marker panel. Kaplan-Meier TDM curves of patients grouped into quartiles based on the cross-validated prediction score of the four-marker panel. *Left*, results for full data set (C index = 0.668; $P < 0.01$), *right*, subgroup of patients who received adjuvant endocrine therapy. P value based on log-rank test null distribution as determined in Fig. 2D.

consolidates its role in breast cancer molecular classification. Whether and how PITX2 gene products are involved in breast carcinogenesis and progression is, however, still unclear.

In addition to PITX2, we generated DNA methylation profiles of regulatory regions of 18 candidate genes, which have been earlier linked to the PITX2 signal transduction pathway. Remarkably, eight of these candidate genes (BMP4, LMX1A, BARX1, FGF4, NR5A1, LHX4, ZNF1A1, and CCND2) were, like PITX2, significantly associated with patient outcome in the present study. This observation is in line with our hypothesis that signal transduction pathways related to PITX2 may play a key role in breast cancer progression.

In total, we have identified 15 novel DNA methylation markers, linked to 14 genes, which were significantly associated with TDM in our cohort (Table 2). Furthermore, our study represents the first evidence for DNA methylation of BMP4, FGF4, FOXL2, C20orf55, BARX1, NR51A, NR2E1, and ZNF1A1 in cancer, whereas DNA methylation for LMX1 was described in the context of colorectal cancer (31) and for TLX3 and LHX4 in prostate cancer cell lines (32) as well as in primary lung cancer and melanoma (33), respectively. DNA methylation of the regulatory region CCND2 has been reported in breast cancer (34) and has been associated with worse outcome before in ovarian and prostate cancers (35, 36). Our finding that increased BRCA1 DNA methylation was associated with worse outcome is in line with the report of Egawa et al. (37) and might hint to a prognostic component of BRCA1 in our population of patients who had all received adjuvant anthracyclines. However, this conclusion remains speculative because our study did not contain an anthracycline-free control arm. For PLAU, for which hypomethylation was observed in more aggressive forms of breast cancer (38–41), we analyzed two fragments within the promoter region and found for both fragments evidence for increased DNA methylation and higher risk of recurrence (data not shown). The differences with previous findings may be explained by the fact that our patients all had received chemotherapy. It was reported previously by us that patients with higher PLAU protein levels benefited more from adjuvant chemotherapy (42). The general observation that patients with higher protein expression are expected to show low promoter DNA methylation might then contribute to our observation.

In conclusion, our data provide further evidence for the relevance of DNA methylation analysis for breast cancer molecular classification and that higher DNA methylation scores are mostly associated with more aggressive disease and that the genes identified may be relevant for breast cancer progression.

Given the strong evidence for PITX2 and several novel DNA methylation markers as outcome predictors, we analyzed whether combining them into a marker panel might improve their predictive performance. To prove that a panel outperforms an individual marker, overfitting has to be accounted for to derive an unbiased estimate of prediction accuracy. We have applied a nested cross-validation approach that uses the available data more efficiently than the usual division into training and test set: both feature selection and performance estimation can be done on the full data set while, at the same time, the performance estimates (C index) are unbiased (24, 25). As PITX2P2 methylation was already proven to be clinically relevant in several studies, we focused our search on models that included this marker. We identified a four-marker panel with improved predictive ability compared with PITX2P2 alone consisting of PITX2P2, BMP4, FGF4, and C20orf55 (Fig. 2A; Table 3). For the four-marker panel, the 5-year estimate for TDM for the quartile with highest scores is <50%, whereas that of the lowest scores is well above 85% (Fig. 3). Like PITX2, the four-marker panel provides information in addition to established clinical variables (Table 3). As mentioned above, BMP4 and FGF4 can be affiliated with developmental pathways shared with PITX2. C20orf55, which we identified in a genome-wide profiling experiment for aberrant methylation in breast cancer (data not shown), was recently characterized as a member of the FAM110 protein family. Members of this family localize to centrosomes and spindle poles, which might hint to a role in tumor pathogenesis (43).

Due to the retrospective nature of our study, the cohort also included patients who have not been treated according to current standards. For example, not all patients with steroid receptor-positive tumors received additional adjuvant endocrine therapy, because they were diagnosed before adjuvant endocrine therapy was introduced as the standard

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of care in steroid hormone receptor-positive breast cancer independent of menopausal status in all European countries. Based on this consideration, survival probabilities for patients treated according to current standards can best be estimated using the subgroup of our patients that received adjuvant endocrine therapy. For this subgroup of 104 patients, performance estimates suggest that the four-marker panel might be used to delineate a good prognosis subgroup of patients with a 10-year survival of >90%. For this subgroup of patients, more aggressive combination therapy or dose-dense therapy may not be required; instead, standard anthracycline-based chemotherapy followed by adjuvant endocrine treatment might be the preferable treatment option. In contrast, for those patients with poor outcome according to our marker panel, different adjuvant chemotherapy approaches (e.g., addition of taxanes) or completely anthracycline-free regimens may be warranted because they do not seem to derive substantial benefit from a standard anthracycline-containing regimen. Thus far, a validated marker for anthracycline benefit (topoisomerase IIa) is only available for HER-2-positive disease.[8] Therefore, for the first time, we provide evidence for a clinically useful marker panel that may aid decision-making regarding adjuvant anthracycline therapy, an issue with has recently become increasingly important (3).

We note that ongoing clinical research tries to further optimize adjuvant endocrine therapies and to effectively combine them with other chemotherapy or novel targeted agents. Several clinical trials established the enhanced benefit of aromatase inhibitors regarding DFS compared with tamoxifen for adjuvant treatment of postmenopausal breast cancer (44, 45). These studies indicate that individualization of endocrine therapy may result in further improvement of clinical outcome. With refinement of endocrine therapy, it is conceivable that our four-marker panel may be used to delineate subgroups with a 5- to 10-year survival exceeding 90% as in the cohort described in this study. Therefore, a diagnostic test based on the suggested marker panel could have the potential to reduce overtreatment and avoid unnecessary side effects even in high-risk primary breast cancer.

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Disclosure of Potential Conflicts of Interest

O. Hartmann, D. Dietrich, A. Fassbender, K. Welzel, S. Maier, A. Plum, S. Niemann, and R. Lesche: employees of Epigenomics, a company in the business of commercializing diagnostic tests and which owns the patent to the described work. N. Harbeck, M. Schmitt, S. Eppenberger-Castori, F. Spyratos, and J.A. Foekens: research support from Epigenomics.

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Prognostic significance of methylated *RASSF1A* and *PITX2* genes in blood- and bone marrow plasma of breast cancer patients

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Abstract Free circulating DNA is increased in the serum/plasma of cancer patients, and methylation of certain genes has been found to be characteristic for malignancy. Therefore, we investigated the prognostic value of two promising genes, *PITX2* and *RASSF1A*, in peripheral blood-plasma (PB-P) and bone marrow plasma (BM-P) of breast cancer patients. Peripheral blood and bone marrow samples from patients with primary breast cancer were prospectively collected during primary surgery at the Department of Obstetrics and Gynecology in Innsbruck ($n = 428$) from June 2000 to December 2006. The study has been approved by the ethical committee of the Medical University of Innsbruck. Methylation analysis was performed using MethyLight, a methylation-specific quantitative PCR-method. In univariate survival analysis, methylated *PITX2* in PB-P was found to be a significant indicator for poor overall survival (OAS) and distant disease-free survival (DDFS) ($P = 0.001$ and $P = 0.023$). Methylated *RASSF1A* in PB-P was also an indicator for poor OAS and DDFS ($P = 0.001$ and $P = 0.004$). *RASSF1A* had also significant prognostic potential when determined in BM-P ($P = 0.016$). In multivariate survival analysis methylated *PITX2* and *RASSF1A* in PB-P remained as therapy-independent prognostic factors for OAS ($P = 0.021$, $P < 0.001$). For DDFS only *RASSF1A* in PB-P showed prognostic significance ($P = 0.002$). Methylated *RASSF1A* and *PITX2* in PB-P appear to have promising potential as prognostic markers in clinical use.

Keywords *RASSF1A* · *PITX2* · DNA-methylation · Breast cancer · Prognosis

Introduction

The phenomenon of elevated free circulating DNA levels in the serum/plasma of cancer patients was first shown 30 years ago [1]. A variety of DNA alterations have been reported within the circulating free DNA of cancer patients, including point mutations, microsatellite instability, and losses of heterozygosity [1].

In addition to the molecular genetic alterations described above, the silencing of genes by promoter hypermethylation is a common feature in human cancer [2]. The inhibition of gene expression by DNA methylation plays an important role in the regulation of DNA repair, cell cycle, apoptosis, cell–cell adhesion, metastasis, tissue and organ architecture, and various signaling pathways.

The tumor suppressor gene *RASSF1A* is one of the most frequently inactivated proteins—mostly by inappropriate promoter methylation—ever identified in human cancer. It lacks apparent enzymatic activity but contains a Ras association domain and is potentially an effector of the Ras oncoprotein. *RASSF1A* modulates multiple apoptotic and cell cycle checkpoint pathways. Current evidence supports the hypothesis that it serves as a scaffold for the assembly of multiple tumor suppressor complexes and may relay

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pro-apoptotic signaling [3]. Furthermore, we and others have shown that *RASSF1A* is frequently hypermethylated in breast cancer [4–6], and methylated *RASSF1A* has been demonstrated in patients with ductal carcinomas of the breast, both in cancer tissue and in paired serum DNA samples of these patients [7].

Moreover, *RASSF1A* has been reported to be present in nearly all breast cancer cell fractions, but is rare in the serum of patients with non-neoplastic breast conditions [8].

DNA-methylation of another gene, *PITX2*, a bicoid-related homebox transcription factor, has been shown to be an independent prognostic marker in patients with hormone receptor-positive lymph node-negative breast cancer, implying that tumors with a methylated *PITX2* promoter are more aggressive [9]. Furthermore, DNA-methylation of *PITX2* in tumor tissue has been reported to reliably predict the risk of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients [10]. However, the role of *PITX2* in breast carcinogenesis and progression is unclear. *PITX2* is regulated by WNT/DVL/beta-catenin and hedgehog TGFβ pathways [11]. It is essential for normal development of the pituitary gland, craniofacial region, eyes, heart, abdominal viscera, and limbs. It has also been suggested that it plays a role in regulating regionally specific terminal neuronal differentiation of the ventrolateral thalamus and midbrain [12]. Moreover, it is differentially expressed in pituitary adenomas [13]. Germ line mutations of *PITX2* cause Axenfeld-Rieger Syndrome (ARS) [14], but no association with neoplastic disorders has been reported for ARS. Collectively, these data imply a growth or differentiation control function for *PITX2*, which could contribute to malignancy when out of equilibrium [10].

The mechanisms of free circulating DNA release in the blood are poorly understood. Generally, two models are used to explain this phenomenon. First, cells (tumor cells and surrounding normal cells) are thought to undergo apoptosis and/or necrosis in situ, and their DNA is released in the blood stream. Second, cells are thought to detach and extravasate into the blood stream, where they undergo lysis and release their DNA content. In these two models, macrophages may play an intermediary role in the release of free circulating DNA in the blood stream [1]. In line with this second model, the death of circulating tumor cells (CTCs) has been considered one of the possible sources for free circulating DNA in cancer patients [15, 16]. In metastatic breast cancer the presence of CTCs is a prognostic indicator, and a decrease in CTC levels during therapy may indicate clinical response [17, 18]. Koyanagi et al. [19] have shown a correlation between CTCs, detected by three mRNA markers, and serum tumor-related methylated DNA (*RASSF1A*) in peripheral blood of melanoma patients. Methylated *RASSF1A* DNA in the serum of breast cancer patients has been reported to be a surrogate marker for circulating breast cancer cells [8].

This study was designed to evaluate the prognostic significance disseminated tumor cells (DTCs) in bone marrow which are believed to be associated with poor prognosis in primary breast cancer patients [2, 20–24] (results of this part of the study will be published elsewhere) and to investigate whether *RASSF1A* and *PITX2* DNA-methylation in peripheral blood-plasma (PB-P) and in bone marrow plasma (BM-P) are surrogate markers of DTCs or have prognostic or predictive potential independent of DTCs.

Materials and methods

General study design

Peripheral blood (PB-P) and bone marrow (BM-P) samples from patients with primary breast cancer were prospectively collected during primary surgery at the Department of Obstetrics and Gynecology at our Medical University Hospital in Innsbruck. After DNA isolation and quantitative PCR analysis of the *RASSF1-A* and *PITX2* DNA-methylation by using Methylight, we applied univariate and multivariate survival models to explore the potential of *RASSF1A* and *PITX2* DNA-methylation in predicting distant disease-free (DDFS) and overall survival (OAS) in patients with primary breast cancer. All included patients gave written consent to the use of their PB-P and BM-P for research purposes. The study was approved by the local Institutional Ethics Review Board and it was performed in concordance with the Reporting Recommendations for Tumor Marker Prognostic Studies of the National Cancer Institute [25]. Clinical, pathological, and follow-up data were stored in a database in accordance with our hospital privacy rules.

Patients

All patients for this study were treated at the Department of Obstetrics and Gynaecology of the Innsbruck Medical University, Austria between June 2000 and December 2006 and staged according to the International Federation of Gynaecology and Obstetrics (FIGO) system. We included 428 female patients with primary breast cancer in our study. None of the patients was diagnosed to have a distant metastatic disease at primary surgery. All patients were monitored within the outpatient follow-up program of the Department of Obstetrics and Gynecology, Innsbruck Medical University, and the median observation period of the patients was 51 months (IQR 35–68). Median age of the patients at surgery was 57.4 years (IQR 50.0–66.3).

Follow-up information was available for all patients. The clinicopathological characteristics of the patients included are summarized in Table 1.

Types of primary surgical treatment were: mastectomy (23%), skin sparing mastectomy (13%), and breast-conserving therapy (64%). Neoadjuvant chemotherapy was applied in 13% of patients. Adjuvant therapies were chemotherapy (32%), endocrine therapy (85%), radiotherapy (79%), and anti-HER2 therapy (5%). 12% of the patients received only chemotherapy (34% anthracycline, 56% combined anthracycline/taxane, 10% others), 64% only endocrine therapy. 20% of the study population received chemotherapy and endocrine therapy (33% anthracycline, 53% combined anthracycline/taxane, 14% others). 55% of the patients, who were treated with an endocrine therapy, received tamoxifen (13% in combination with GnRH-Analoga), whereas for 23% of this groups aromatase inhibitors were used.

Specimen characteristics and laboratory work

For bone marrow sampling a bilateral BM aspiration was performed, as described in [20, 26]. 10 ml BM was taken out from each side of the iliac crest/pelvis crest, immediately transferred to a 50-ml Falcon tube (BD Biosciences, USA), mixed with 1 ml EDTA to avoid coagulation and

Table 1 Associations of clinicopathologic characteristics with negative versus positive DNA-methylation in peripheral blood-plasma and bone marrow plasma (χ^2-Statistic)

	n	Peripheral blood-plasma (PB-P)		Bone marrow plasma (BM-P)	
		PITX2 (*P*-value)	*RASSF1A* (*P*-value)	*PITX2* (*P*-value)	*RASSF1A* (*P*-value)
Age					
<57.4	214	0.763	<0.001	0.135	<0.001
≥57.4	214				
Menopausal status					
Pre	131	0.870	0.009	0.012	<0.001
Post	292				
Unknown	5				
pT					
pT-0	9	0.511	0.237	0.784	0.921
pT-1	318				
pT-2/pT-3	77				
Unknown	25				
LN					
Negative	275	0.318	0.679	0.333	0.290
Positive	141				
Unknown	12				
Grade of malignancy					
1	76	0.098	0.108	0.054	0.899
2	292				
3	55				
Unknown	5				
Estrogen receptor					
Negative	66	0.662	0.586	0.025	0.612
Positive	358				
Unknown	4				
Progesterone receptor					
Negative	76	1.0	0.405	0.427	0.148
Positive	348				
Unknown	4				
Her-2					
Negative	277	0.726	0.773	0.504	0.055
Positive	124				
Unknown	27				
Total	428				

neg negative, *pos* positive, *pT* pathologic tumor staging, *LN* lymph nodes

pooled afterwards. Blood samples were collected in 10 ml EDTA containing collection tubes (S-Monovette, Sarstedt, Germany). Both, PB and BM samples were either worked up immediately or kept at 4°C (no longer than 4 h) before processing. Estrogen and progesterone receptor levels were determined in patient paraffin sections by immunohistochemistry with a DAKO Autostainer. The following primary antibodies were used: monoclonal Mouse Anti-Human Estrogen-Receptor α (DAKO, clone 1D5, dilution 1:100); and Monoclonal Mouse Anti-Human Progesterone-Receptor (DAKO, clone PgR 636, dilution 1:200). Chemmate DAKO Envision™ K5007 was used as the secondary antibody (DAKO, Glostrup, Denmark). Receptor status was recorded as a percentage of stained cells (0–100%) and staining intensity (1–3) for both ER and PR. Samples with a percentage >10% staining were considered receptor-positive. HER2/neu status was analyzed immunohistochemically using the HercepTest (DAKO, Glostrup, Denmark). Membrane immunoreactivity and membrane staining patterns were evaluated and scored using the 0 to 3+ scoring system according to the manufacturer's protocol. Tumors with a HER-2/neu score of 2+ in immunohistochemical analysis were also tested for HER-2/neu gene amplification by fluorescence in situ hybridization (FISH) using the PathVysion kit (Vysis, Downers Grove, IL, USA). According to the HER-2/neu testing recommendations of the American Society of Clinical Oncology and the College of American Pathologists, tumors were classified as positive when the immunohistochemical score was 3+ or gene amplification was detected by means of FISH [27].

DNA isolation

Blood samples were centrifuged at 1000×*g* for 5 min at room temperature. Bone marrow samples were centrifuged at 500×*g* for 5 min; supernatant was taken and again centrifuged at 1000×*g* for 5 min at room temperature. 1 ml aliquots of PB-P and BM-P were stored at −50°C.

Genomic DNA from plasma and bone marrow supernatant was isolated using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol with some modifications, as already published [8].

Analysis of DNA methylation

Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Sodium bisulfite-treated genomic DNA was analyzed by MethyLight, a fluorescence-based, quantitative real-time PCR assay, as described previously [6]. In brief, a set of primers and probe, designed specifically for bisulfite-converted DNA, was used for the genes of interest, *RASSF1A* and *PITX2*. Primer and probe sequences were as follows: *RASSF1A* forward primer, 5′-ATTGAG TTGCGGGAGTTGGT-3′; *RASSF1A* reverse primer, 5′-A CACGCTCCAAC CGAATACG-3′; *RASSF1A* probe, 5′-F AM-CCCTTCCCAACGCGCCCA-BHQ1-3′, according to Fiegl et al. [8]; *PITX2* forward primer, 5′-AGTTCGGTT GCGCGGTT-3′; *PITX2* reverse primer, 5′-TACTTCCC TCCCCTACCTCGTT-3′; and *PITX2* probe, 5′-FAM-C GACGCTCGCCCGAACGCTA-BHQ1-3′, sequences for *PITX2* primers and probe were provided by Epigenomics AG, Berlin, Germany. For each MethyLight reaction 10 µl of bisulfite-treated genomic DNA was used.

Interassay variance of the MethyLight reactions and the whole procedure (including DNA isolation and sodium bisulfite conversion) was determined using SssI treated (completely methylated) human white blood cell DNA (New England Biolabs, Ipswich, MA, USA) and control-plasma (CP) from a healthy volunteer that was supplemented with DNA from diverse breast cancer cell lines (MCF-7, T-47D, MDAMB231 and ZR75-1). Variance was defined as a 90% range of Ct-values for each gene at the median Ct-value. For comparison we calculated the corresponding variation coefficient. All of these values are shown in Table 2. Specificity and accuracy of MethyLight reactions was controlled using SssI completely methylated human white blood cell DNA as a positive control and distilled water as a negative control.

Real time PCR for RASSF-1 was performed within 36 h after bisulfite modification. qPCR for PITX-2 was performed one half to one year after bisulfite modification, the samples being stored at −80°C. It could be proved with 10 different samples that the storage under above conditions had no influence at all on the result of qPCR (data not shown). The thermal cycling conditions for RASSF-1 and PITX-2 comprised an initial denaturing step at 95°C for 10 min and 50 cycles of 95°C for 15 s and 60°C for 1 min.

Patients' samples were analyzed in triplicate. A patient sample was defined as positive when the minimum Ct-value of the triplicates was ≤40. As we were interested in the absolute amounts of methylated DNA, we did not normalize results to a reference gene. Results were only related to the deployed serum volume as this was kept constant.

Bone marrow preparation and ICC

The procedure for bone marrow preparation and ICC has been described previously [9]. In short, bone marrow aspirates were separated by Ficoll density-gradient centrifugation to enrich mononuclear cells. Mononuclear cells were removed from the interface, washed, and cytospin

Table 2 Interassay variance of MethyLight reaction only (using treated SssI) and the entire method including DNA isolation, sodium bisulfite conversion and MethyLight reaction (using control plasma)

Control	Gene	Median Ct-value	90% Range of variation	Variation coefficient
SssI treated DNA	Methylated *PITX2*	35.51	0.85	18.5%
SssI treated DNA	Methylated *RASSF1A*	35.04	1.31	26.4%
Control plasma	Methylated *PITX2*	29.91	1.71	47.2%
Control plasma	Methylated *RASSF1A*	27.57	1.37	30.2%

SssI treated (completely methylated) human white blood cell DNA standard

slides were prepared at 1×10^6 MNC/slide. They were air-dried overnight at room temperature, fixed in 3.7% formalin and either immunostained directly or stored at −80°C until immunostaining was performed. For immunohistochemical staining we used monoclonal antibody A45-B/B3 (Micromet, Munich, Germany), which is directed against a common epitope on cytokeratin polypeptides, including the cytokeratin heterodimers 8–18 and 8–19. The reaction of the primary antibody was developed with the alkaline phosphatase anti-alkaline phosphatase technique combined with the new fuchsine stain to indicate antibody binding, as previously described [9]. Evaluation of ICC was carried out by the Automated Cell Imaging System ACIS (ChromaVision GmbH, Karlsruhe, Germany) and verified by our pathologist. For each patient a total of 4×10^6 mononuclear cells were screened for the presence of DTCs. To identify cells we not only used immunocytochemical staining but also morphologic features according to Borgen et al. [28].

Statistical analysis

Associations between *RASSF1A* and *PITX2* methylation status in PB-P and BM-P and clinicopathologic characteristics of the patients were assessed with Pearson's chi-square test. DDFS was defined as the time from surgery to histopathological confirmation of distant metastases or death. OAS was defined as the time from surgery to death from any cause or to the last clinical inspection. Univariate survival analyses were conducted using the Kaplan–Meier method, and differences between groups were determined with the log-rank test. A Cox proportional hazards regression model with stepwise backwards variable-selection was applied for multivariate survival analyses. Variables with $P < 0.05$ in the univariate survival analysis were included into the multivariate model.

In sensitivity analysis we further investigated the effect of *RASSF1A* and *PITX2* methylation status on the above endpoints, modeling the methylation status of *RASSF1A* and *PITX2* as a continuous variable, using penalized splines (P-splines) in extended, restricted maximum-likelihood (REML-) optimal Cox-type additive hazard regression [29].

A P-value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (Version 15.0 for Windows).

Results

DNA methylation status of *RASSF1A* and *PITX2* was assessed in PB-P and BM-P of primary breast cancer patients. The prevalence of methylated *RASSF1A* was 21.8% in PB-P and 20.6% in BM-P. For methylated *PITX2* it was 13.9% in PB-P and 44.2% in BM-P. *RASSF1A* or *PITX2* methylation was found in 30.7% of PB-P samples and 47.6% of BM-P samples. Unexpectedly, methylated *PITX2* and *RASSF1A* were also observed in healthy controls (data not shown).

DNA methylation levels of *RASSF1A* and *PITX2* were significantly associated in PB-P ($P = 0.005$) and BM-P ($P < 0.001$). Significant association was also observed between PB-P and BM-P for both *RASSF1A* ($P < 0.001$) and *PITX2* ($P = 0.009$).

We compared the analyzed markers with clinical indicators for prognosis: stage, age, grade of malignancy, menopausal status, HER-2 expression, ER, PR, lymph node status, and the presence of DTCs. A significant association was observed between methylation status of *RASSF1A* (in PB-P and BM-P) and age (<median vs. >median) as well as menopausal status ($P < 0.002$ for all). *PITX2* methylation was associated with ER and menopausal status in BM-P. Neither *RASSF1A* nor *PITX2* in both PB-P and BM-P showed any association with the presence of DTCs.

In univariate Kaplan–Meier survival analyses, methylation of *RASSF1A* as well as *PITX2* in PB-P was shown to be a prognostic factor for OAS and DDFS ($P = 0.001$ and 0.023 resp.). Combining the methylation status of both genes in PB-P lead to a higher proportion of positive results and also to a higher prognostic significance for OAS and DDSF ($P < 0.001$ for both). Kaplan–Meier curves for *PITX2* and *RASSF1A* are shown in Fig. 1. All other information about univariate survival analysis is provided in Tables 3 and 4. Neither *PITX2* nor *RASSF1A* showed prognostic relevance in patients who received only chemotherapy. In patients treated with adjuvant hormone therapy methylated *RASSF1A* in PB-P was shown to be a

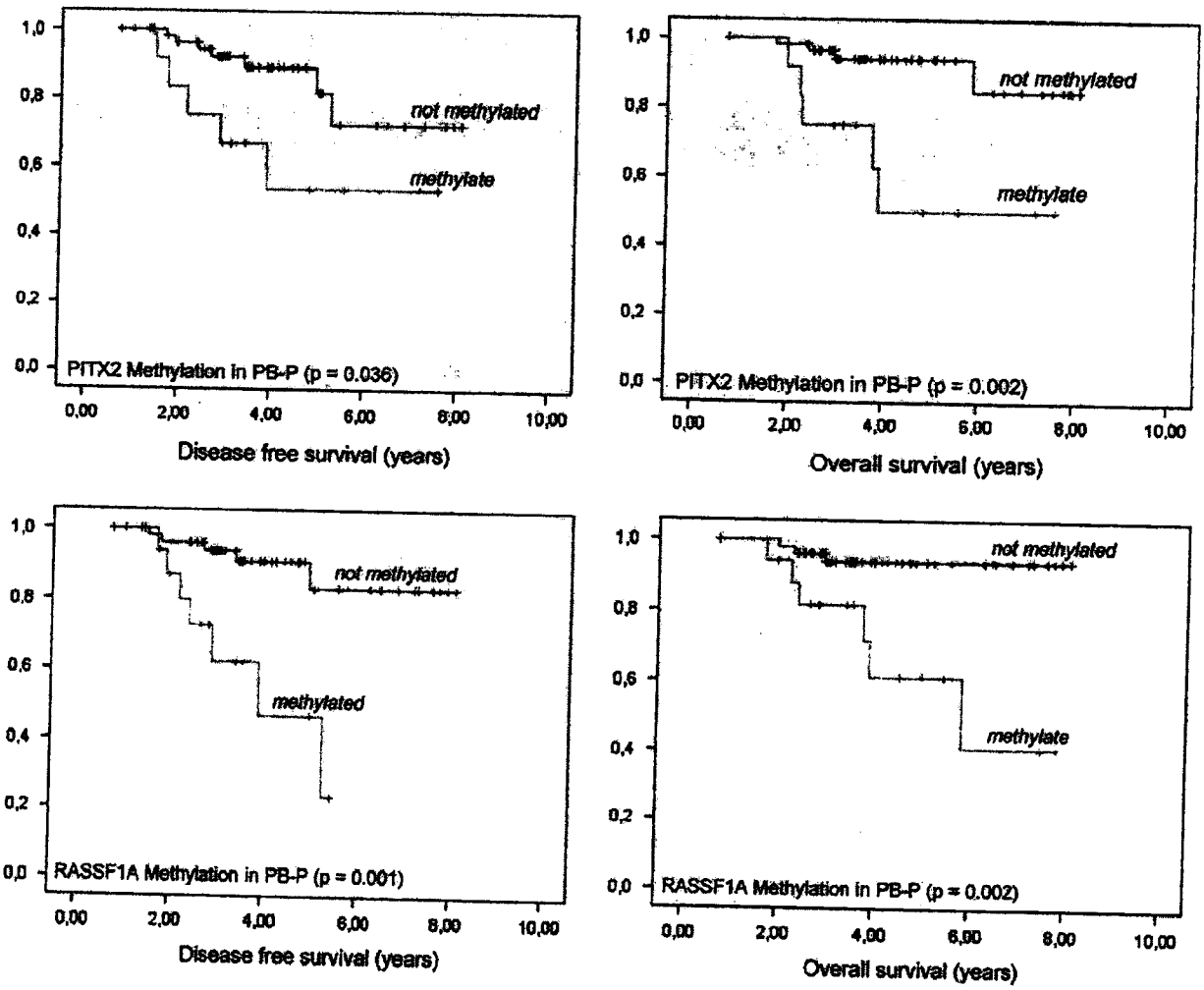

Fig. 1 Univariate Kaplan–Meier survival analysis of patients with low/high RASSF1A and PITX methylation in peripheral blood-plasma (PB-P). *P*-values indicate results of the log-rank test

prognosticator for poor OAS and DDFS ($P = 0.035$ and 0.008). Furthermore methylated *PITX2* and *RASSF1A* in PB-P were associated with significantly lower OAS and DDFS in patients who received combined chemo-hormone-therapy (DDFS: $P = 0.036$, $P = 0.001$; OAS: $P = 0.002$ for both *PITX2* and *RASSF1A*). In this group also patients with methylated *RASSF1A* in BM-P showed lower OAS and DDFS ($P = 0.017$ and $P = 0.002$).

In a multivariate time-independent Cox regression adjusted for stage, age, grade of malignancy, menopausal status, Her-2 expression, ER, PR, and lymph node status, methylated *PITX2* in PB-P and methylated RASSF1 in PB-P remained as independent prognostic factors for OAS whereas for DDFS only *RASSF1A* methylation in PB-P together with LN- and ER-status had significant impact. Further details are shown in Table 5.

Analyzing the associations of *PITX2* and *RASSF1A* methylation status with OAS and DDFS, using *PITX2* and *RASSF1A* methylation status as a continuous covariate in a flexible, univariate, and multivariate non-parametric P-spline regression model widely confirmed the results of the main multivariate Cox model with *PITX2* and *RASSF1A* as dichotomized variables.

Discussion

Numerous prognostic factors have been described for breast cancer. However, many of them lack practicability, as they are determined in fresh/frozen tissue, which is not available in low stage tumors. Prognostic parameters analyzed in a patient's blood would therefore be much more

Table 3 Kaplan–Meier survival analysis-1 (log-rank test)

	OAS			DDFS		
	P-value	5-year survival Positive	5-year survival Negative	*P*-value	5-year survival Positive	5-year survival Negative
Peripheral blood-plasma						
Methylated *PITX2* ($n = 359$)	0.001	80.1% ($n = 50$) ($n^e = 10$)	95.1% ($n = 309$) ($n^e = 15$)	0.023	79.1% ($n = 50$) ($n^e = 10$)	91.1% ($n = 309$) ($n^e = 25$)
Methylated *RASSF1A* ($n = 357$)	0.001	85.8% ($n = 78$) ($n^e = 13$)	95.4% ($n = 279$) ($n^e = 12$)	0.004	80.6% ($n = 78$) ($n^e = 15$)	91.3% ($n = 279$) ($n^e = 21$)
Methylated *PITX2* or *RASSF1A* ($n = 355$)	<0.001	85.6% ($n = 109$) ($n^e = 18$)	96.6% ($n = 246$) ($n^e = 7$)	0.001	82.3% ($n = 109$) ($n^e = 20$)	92.4% ($n = 246$) ($n^e = 15$)
Bone marrow plasma						
Methylated *PITX2* ($n = 406$)	0.481	91.4% ($n = 179$) ($n^e = 16$)	93.9% ($n = 227$) ($n^e = 12$)	0.718	89.4% ($n = 179$) ($n^e = 19$)	88.8% ($n = 227$) ($n^e = 21$)
Methylated *RASSF1A* ($n = 403$)	0.016	87.8% ($n = 83$) ($n^e = 11$)	94.1% ($n = 320$) ($n^e = 17$)	0.087	87.3% ($n = 83$) ($n^e = 13$)	89.8% ($n = 320$) ($n^e = 27$)
Methylated *PITX2* or *RASSF1A* ($n = 403$)	0.239	91.5% ($n = 211$) ($n^e = 19$)	94.1% ($n = 192$) ($n^e = 9$)	0.767	89.8% ($n = 211$) ($n^e = 22$)	88.0% ($n = 192$) ($n^e = 18$)

OAS overall survival, *DDFS* distant disease-free survival, n number of cases, n^e number of events

Table 4 Univariate Kaplan–Meier survival analysis stratified by the type of adjuvant therapy (log-rank test)

	Chemotherapy $n = 51$		Hormone therapy $n = 275$		Chemotherapy + hormone therapy $n = 83$	
	DDFS	OS	DDFS	OS	DDFS	OS
Methylated *PITX2* in PB-P	0.454	0.543	0.516	0.187	0.036	0.002
Methylated *RASSF1A* in PB-P	0.925	0.801	0.035	0.008	0.001	0.002
Methylated *PITX2* in BM	0.872	0.633	0.458	0.446	0.778	0.770
Methylated *RASSF1A* in BM	0.490	0.532	0.939	0.257	0.017	0.002

PB-P peripheral blood-plasma, *BM* bone-marrow, *OAS* overall survival, *DDFS* distant disease-free survival

convenient. Disseminated tumor cells were analyzed as a prognostic factor in our breast cancer center. Because this analysis was performed in bone marrow, we were able to investigate the two markers in both peripheral blood and in the corresponding bone marrow. Free circulating DNA in serum and plasma has been shown to be increased in cancer patients [1, 30]. Therefore, the analysis of this DNA fraction might be a useful tool to characterize a tumor.

It was interesting to note that the prevalence of positive results was higher in BM-P than in PB-P for methylated *PITX2*, but not for methylated *RASSF1A*, although these two markers correlate significantly with each other in PB-P as well as in BM-P. This indicates that *PITX2* and *RASSF1A* are not co-expressed in tumor cells—or are methylated in a different manner—or that they derive from different cell types. Assuming that methylated *PITX2* and *RASSF1A* occur exclusively in tumor cells, methylated *PITX2* would be the more sensitive marker for breast cancer cells. However, the fact that patients with benign lesions of the breast also show positive results for methylated *PITX2* and *RASSF1A* speaks against considering them classic tumor markers, as both sensitivity and specificity are low. Occurrence of methylated *PITX2* and *RASSF1A* in benign conditions speaks against the concept

Table 5 Therapy-independent significant prognostic factors for OAS and DDFS

Factor	Hazard ratio	95%CI	*P*-value
OAS			
Methylated *PITX2* in PB-P (low vs. high)	3.4	1.2–9.8	0.021
Methylated *RASSF1A* in PB-P (low vs. high)	5.6	2.1–14.5	<0.001
DDFS			
Methylated *RASSF1A* in PB-P (low vs. high)	3.4	1.6–7.3	0.002
LN (neg. vs. pos)	2.5	1.1–5.8	0.036
ER (neg. vs. pos)	0.3	0.1–0.9	0.039

Time-independent multivariate Cox Regression Model adjusted for clinicopathological factors and stratified by the type of therapy (backward selection of variables)

LN lymph-nodes, *PB-P* peripheral blood-plasma, *OAS* overall survival, *DDFS* distant disease-free survival, *ER* estrogen receptor, *CI* confidence interval

that they are malignancy-specific, at least concerning the serum fraction. It could be speculated that in all cases where increased cell turn over occurs, methylated *PITX2* and *RASSF1A* appear in the serum. Associations of analyzed markers with clinical indicators of prognosis were observed for *RASSF1A* (in PB-P and BM-P) and for *PITX2* in BM-P, but only with age and menopausal status. *PITX2* in PB-P did not correlate with any of the clinical parameters. This speaks for an essential influence of the host on the methylation of these two genes. Kaplan–Meier survival analysis indicated a variety of significant differences for OAS and DDFS for both markers. Results in BM-P were only significant for *RASSF1A* methylation and OAS. In the different treatment groups the strongest differences occurred in the group of combined chemo-hormone-therapy. This could mean that the significant differences obtained when analyzing the whole population of patients may derive mainly from this group of patients. In a multivariate analysis *PITX2*- and *RASSF1A*-methylation in PB-P remained independent prognosticators. This implies, together with the results of univariate analysis, that PB-P is superior to BM-P as a sample source for the determination of these two biomarkers.

When comparing the prognostic potential of methylated *PITX2* and *RASSF1A*, it is not clear which one of them allows better discrimination between good and bad prognosis. A combination of both markers seems to improve the diagnostic validity. The great advantage of these markers is that they can be determined in blood, which would represent a step forward in the evaluation of the course of disease in breast cancer patients.

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DNA methylation as a biomarker in breast cancer

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In cancer, epigenetic changes such as covalent addition of methyl groups to the genomic DNA itself are more prominent than genetic changes. Cytosine–phosphate–guanosine (CpG) methylation negatively affects gene transcription of an adjacent gene. It is thought that DNA methylation significantly contributes to all hallmarks of cancer. Next to being a potential therapy target, DNA methylation is an emerging field of biomarkers. Technically, DNA provides a stable and robust analyte that is particularly suitable for clinical applications. Moreover, there are numerous potential human DNA sources that could facilitate integration of methylation tests in clinical practice. In breast cancer, DNA methylation has shown promise as a potential biomarker for early detection, therapy monitoring, assessment of prognosis or prediction of therapy response. In particular, paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation has been validated using a robust assay for paraffin-embedded tissue for clinically relevant outcome prediction in early breast cancer patients treated by adjuvant tamoxifen therapy.

Review
Future Oncology

Biomarkers & breast cancer

Breast cancer is a prevalent human malignancy and a very common cause of cancer-related death among women worldwide. Over the last few decades, early detection and novel treatment strategies have improved survival rates quite significantly in breast cancer patients. The most valuable prognostic factors in early breast cancer are still the presence and number of axillary lymph node involvement, as well as tumor size. However, these established prognostic factors are becoming clinically less useful. With increased early detection efforts, tumors are getting smaller and the percentage of node-negative tumors is increasing. It is thus obvious that biomarkers that can reliably forecast disease progression are urgently needed. The same is true for accurate predictors of the risk of breast cancer and of response to various treatment modalities, as well as biomarkers for monitoring during therapy or after surgery. Traditionally, most tumor markers are proteins that are measured in either serum, plasma (mostly by enzyme-linked immunosorbent assay [ELISA]) or in tumor tissue (by ELISA or immunohistochemistry) [1].

With regard to clinically useful prognostic factors, only very few of the biomarkers proposed in the literature have successfully passed the strict evidence-based criteria for clinical utility in breast cancer [2]. In addition to serum tumor markers CA 15-13, CA 27-29 and carcinoembryonic antigen (CEA), as well as tissue-based markers steroid hormone receptors (estrogen receptors [ERs], progesterone receptors [PRs]) and HER2 status, among so-called new molecular tests, only urokinase plasminogen activator (uPA), plasminogen activator inhibitor (PAI)-1 and the 21-gene recurrence score have been recommended by the American Society of Clinical Oncology (ASCO) in 2007 for routine clinical application [3].

Whereas the serum-based tumor markers are recommended for monitoring of therapy response in the advanced setting, tumor-associated ER, PR and HER2-status are predictive regarding therapy response to endocrine or anti-HER2 targeted therapy, respectively. uPA/PAI-1, two protein markers determined in tumor tissue extracts, indicate tumor aggressiveness and thus are able to forecast disease outcome. These markers have been validated at the highest level of evidence (LOE I) by the prospective, randomized Chemo N0 therapy trial [4], as well as the European Organisation for Research and Treatment of Cancer (EORTC) PathoBiology Group (PBG) pooled analysis in more than 8000 patients [5], for clinical decision making in node-negative breast cancer. The 21-gene recurrence score has been retrospectively validated as a marker that predicts outcome in hormone receptor-positive disease having received adjuvant endocrine therapy [6]. Similarly, a number of multigene assays (e.g., Amsterdam 70-gene signature [7], Rotterdam 76-gene signature [8], the HOXB13/IL17BR ratio [9] genomic grade [10] and so on) have retrospectively been shown

Keywords

breast cancer ▪ DNA methylation ▪ microarray ▪ PITX2 ▪ prognosis ▪ uPA/PAI-1

to provide additional prognostic information in primary breast cancer. As a result, the 21-gene recurrence score is currently being prospectively evaluated in the Trial Assigning Individualized Options for Treatment (TAILORx) [11], while for the 70-gene signature a prospective validation is performed in the international Microarray in Node-Negative Disease May Avoid Chemotherapy (MINDACT) trial [12]. Next to a lack of prospective clinical validation, technical issues such as sampling and storage of tissue specimens, as well as reliable prospective quality assurance and multicenter determination possibilities, in particular for RNA-based assays, are one explanation why only two of the so-called new molecular tests have so far been recommended by ASCO for clinical routine use.

Epigenetics

The term epigenetics refers to heritable phenotypic changes, which are not the result of alterations in the nucleotide sequence of the genome. In cancer, epigenetic changes are more prominent than genetic changes, and thus although cancer is a genetic disorder, it is evident that epigenetic alterations significantly contribute to this dreadful disease [1,13,14]. It is, therefore, not surprising that the cancer epigenome is a target for development of novel cancer treatments [14–17]. Two main types of epigenetic changes exist, which both involve covalent modification of the chromatin. The first type involves acetylation, methylation and other types of histone modifications, while the second type involves covalent addition of methyl groups to the genomic DNA itself [14]. Both types of modifications are aberrant in cancer and adversely affect tumor cell behavior [13,14]. The latter type of epigenetic alteration is the focus of this review, and its role in cancer is currently studied in great detail and is so far also better understood. DNA methylation occurs in mammals only at cytosines on the fifth residue of its base, and this methylcytosine nucleotide is often referred to by epigeneticists as 'the fifth base'. 5-methylcytosines are only observed upstream of guanine residues in so-called cytosine–phosphate–guanosine (CpG) dinucleotides. CpG methylation negatively affects gene transcription of an adjacent gene [17–19], and is therefore considered, next to gene mutation or gene loss, as another means to silence a gene and thus a nongenetic means to satisfy Knudson's tumor suppressor gene hypothesis [16,20]. There is a negative selection against methylated CpGs as they are sensitive for genetic aberrations. Still, CpGs are retained in foreign and repetitive DNA and in centrosomal regions of the genome. The idea is that CpGs in these genomic regions – which are generally methylated – conserve the integrity of the genome, as it prevents chromosomal regions from being engaged in recombination during repair, mitosis and meiosis. Furthermore, CpG methylation in such regions prevents foreign genetic material from being transcribed. Apart from these intergenic regions, various imprinted regions of the genome and almost one entire copy of X-chromosome in females is also methylated on CpGs. In human tumors, global hypomethylation of intergenic regions was initially observed [14,21–24]. Soon thereafter, and probably more relevant for cancer progression, it was revealed that transcription start sites of many genes were hypermethylated on CpGs. Initially, hypermethylation of CpGs sites grouped together in so-called CpG islands was seen in known tumor-suppressor genes [14,21–24], but more recently polycomb-regulated genes involved in cellular differentiation were also frequently hypermethylated in their promoter [25]. Why certain genes become methylated in cancer is still not understood. However, one pattern that emerges is that tissue-specific polycomb-regulated genes become progressively methylated in cancer (epigenetic switching), presumably preventing tumor cells from differentiating and thereby retaining their proliferative potential [25,26]. Combined with *de novo* methylation of classical tumor suppressor genes via a still poorly understood mechanism termed 'methylation reprogramming', it is thought that DNA methylation significantly contributes to all hallmarks of cancer [26].

DNA methylation as a biomarker

DNA methylation has interesting features that suggest its use as a cancer biomarker. First, DNA methylation is relatively stable compared with most other biomarkers, and accessible from routinely collected formalin-fixed paraffin-embedded (FFPE) clinical material. In addition, the epigenetic tag is localized to CpG islands near gene initiation sites, and after bisulfite conversion, it is amplifiable as any other nucleic acid-based biomarker. In the previous century, discovery of epigeneric biomarkers, as for every biomarker investigated around that time, involved screening of newly discovered tumor suppressor genes for their epigenetic silencing through DNA methylation. This screening showed that many known tumor suppressor genes show aberrant DNA methylation, which is often mutually exclusive with gene mutation and/or loss of heterozygosity

(LOH), thereby underscoring that epigenetic silencing of genes through DNA methylation is a valid way of abrogating gene function next to genetic gene inactivation. As a result, mounting evidence suggests that hypermethylation may be the most prevalent phenomenon in human malignancies, and thus could prove to be a useful cancer biomarker for clinical applications. Among others, methylation-specific polymerase chain reaction (MSP), quantitative PCR-based methods such as bisulfite treatment in combination with MethyLight [27] or pyrosequencing [28], high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) are quick and sensitive techniques to detect DNA hypermethylation from a wide variety of blood and tissue sources [1,29–31]. MSP requires only small quantities of DNA, has high specificity and is sensitive enough to identify one methylated allele among 1000 unmethylated alleles [32,33]. Around the turn of the century when the human genome was completed, the field was revolutionized when medium- to high-throughput technologies entered the arena. Currently, whole-genome analyses of methylation are feasible by specific immunoprecipitation of methylated CpGs (MeDIP) followed by hybridization on whole-genome tiling arrays or by ultra-deep sequencing of the DNA tags using current next-generation sequencing machines [34].

Potential DNA sources for methylation biomarker detection

The number of potential human DNA sources is relatively large, which could greatly facilitate integration of methylation tests in routine clinical practice [35]. Cancer-specific DNA hypermethylation patterns have been detected in FFPE tumor specimens, fresh–frozen tumor samples, exfoliated luminal tumor cells, cellular DNA and free-tumor DNA from plasma, ductal lavage from the breast, as well as a variety of body fluids including urinary sediment, saliva, sputum, bronchial washings and ejaculate [32,36–39]. Interestingly, noncellular circulating free DNA (cir-DNA; also known as cell-free plasma DNA [cfp-DNA] or cell-free DNA) has been shown to contain comparable tumor-specific alterations as DNA from tumor tissue. The cir-DNA has also been found on the surface of circulating blood cells, which may help increase the sensitivity of these type of methylation assays [40–42]. The origin of cir-DNA remains unknown, but it could be released from tumor cells that succumbed to apoptosis or necrosis. Cir-DNA is present in clinically healthy individuals at a concentration of approximately 10 ng/ml [43]. The observation that cir-DNA is more abundant in several body fluids from cancer patients than in those from healthy controls suggests that detection of cir-DNA can potentially be used for cancer screening for early diagnosis [32]. In breast cancer, ductal lavage can provide sufficient material for cellular and cirDNA analysis and cytological diagnosis through the safe and minimally invasive sampling of breast ductal fluid [32].

Clinically relevant DNA methylation biomarkers

In recent years, it has become clear that DNA hypermethylation of specific genes may become a useful biomarker in the clinical setting (Figure 1), for example, to identify individuals at increased risk of developing cancer or for screening of asymptomatic individuals, both facilitating early cancer diagnosis. DNA methylation markers could also be used to establish disease prognosis or guide tailored treatment. Lastly, detecting epigenetic markers in serum may be valuable for monitoring cancer therapy or assessing disease progression [1,14,35]. This review only focuses on recently discovered clinically significant epigenetic biomarkers involving DNA methylation in breast cancer that we and others have recently discovered (Figure 1).

Screening, early diagnosis & prediction of disease progression by DNA methylation biomarkers

Considering that current serum-based biomarkers, such as CA15–3, are of little value in detecting breast cancer in asymptomatic patients [3], aberrant hypermethylation of specific CpG islands, being uncommon in normal cells and an early event in cancer development, is a good alternative to detect patients at risk of developing breast cancer and could have diagnostic applicability in carriers with high-penetrance mutations in disease-causing tumor-suppressor genes [40]. For example, identification of DNA hypermethylation in the serum of a *BRCA1* mutation carrier could be useful for screening purposes in yet unaffected mutation carriers [15]. Acquiring DNA for methylation assays from archived serum specimens may be useful in early detection screening, especially in subjects with a family history of cancer [15,29]. *BRCA1* gene hypermethylation appears to be tumor-specific as it is not detectable in normal breast tissue. So far, *BRCA1* promoter hypermethylation has been detected almost exclusively in sporadic breast and ovarian cancers (except for an isolated case with sporadic lung cancer) [44,45].

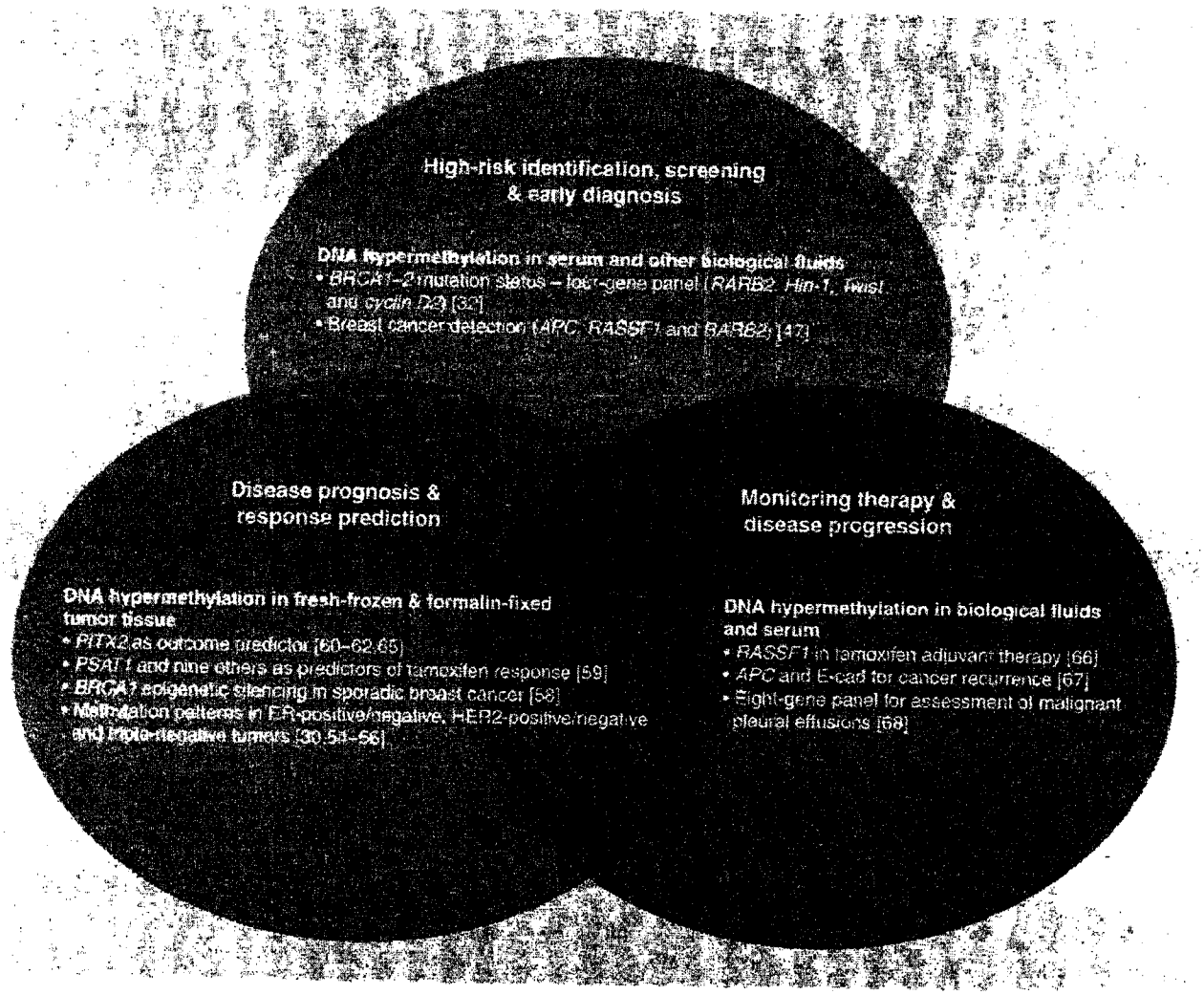

Figure 1. Potential clinical application of DNA methylation tests in breast cancer.

The highest rate of methylation reported thus far is in the promoter region of 14-13-3σ (96% of breast carcinomas and in 38% of atypical hyperplasias [24,46]), but, as reported by Feng *et al.*, this gene is highly methylated in both malignant and paired normal breast tissue, making it unsuitable for development of a screening or diagnostic methylation test [30].

Locke *et al.*, using cir-DNA extracted from ductal lavage fluid from healthy women with known *BRCA* mutation status, proposed the use of a four-gene panel (*RAR*-β, *HIN-1*, Twist and *cyclin D2*) as a surrogate marker for *BRCA1/2* mutation status and for breast cancer risk assessment [32]. The authors found that 42% of *BRCA* mutation carriers had at least one hypermethylated gene in the four-gene panel, while no aberrant hypermethylation was found in ductal lavage samples from five healthy controls ($p = 0.13$).

Serum DNA methylation analysis could offer relatively high sensitivity in diagnosing breast cancer. Hoque *et al.* compared the plasma DNA methylation status of *APC*, *GSTP1*, *RASSF1A* and *RARβ2* from women with breast cancer and healthy controls and reported 62% sensitivity and 81% specificity [47]. Dulaimi *et al.* found a 76% methylation rate in serum compared with 94% in breast tumor tissue when one or more of several genes (*RASSF1*, *APC* and *DAPK*) were examined [48].

Taken together, these preliminary findings suggest that DNA-methylated genes, in combination with mammography, could be useful in screening for breast cancer. Women with negative mammograms but DNA hypermethylation of some particular genes could be identified as being at increased risk of developing breast cancer, and thus undergo a more intensive clinical or

imaging follow-up such as magnetic resonance imaging (MRI) investigations [1]. Unfortunately, for breast cancer, a suitable gene or set of genes consistently hypermethylated in cancer but not in healthy tissue and detectable in serum or plasma have yet to be found.

DNA methylation markers as prognostic & predictive factors in breast cancer

Steroid hormone receptors & growth factors

DNA hypermethylome profiles have been proposed as indicators of disease outcome or predictors of treatment response in breast cancer patients [14]. In clinical practice, ER, PR and HER2 status are recognized as prognostic, and even more importantly, predictive factors in patients with breast cancer, proving most useful for response prediction regarding endocrine-based therapy, such as tamoxifen or aromatase inhibitors, and anti-HER2 targeted therapies such as trastuzumab or lapatinib [49]. On the basis of microarray molecular profiling of invasive breast carcinomas, five distinct tumor subtypes (luminal A, luminal B, normal breast-like, HER2 overexpressing and basal) have been identified that are associated with different clinical outcomes [50,51]. In a similar manner, several prognostic genes and, more recently, also miRNA-expression signatures [6–8,52] have been identified and these have, in most cases retrospectively, been validated. Thus far, this type of profiling has interrogated the transciptome known for its innate instable nature and this type of profiling is less compatible with FFPE tumor tissue, which is generally collected during routine histopathology [53]. Widschwendter *et al.* explored the use of DNA methylation markers as an alternative approach to gene-expression profiling and found, amongst a panel of 35 markers, that the best predictor of ER status was methylation of the *PR* gene [54]. Conversely, the best predictor of PR status was methylation of *ER*. More recently, Sunami *et al.* [55] examined the promoter methylation status of eight major breast cancer-related genes (*RASSF1A*, *CCND2*, *GSPT1*, *TWIST*, *APC*, *NES1*, *RARb2* and *CDH1*) searching for epigenetic differences related to ER and HER2 status in FFPE primary tumors samples. They found several epigenetic differences between ER-positive and -negative and between HER2-positive and -negative breast tumors, as well as between ER-negative/HER2-negative and breast tumors expressing either HER2 or ER. Triple-negative breast tumors, that is those with negative ER, PR and HER2 status, have lately been recognized as an important subgroup of breast cancer with distinct outcome and that are in need of a different therapeutic approach. Triple-negative breast tumors comprise primarily, but not exclusively, a molecularly distinct subtype of breast cancer, the basal-like subtype [56]. Feng *et al.*, using pyrosequencing as quantitative analysis of DNA methylation, described two panels of genes (*HIN-1/RASSF1A*, and *RIL/CDH13*), which correlated, either positively or negatively, with ER or PR status [30]. The subset of triple-negative breast cancers was positively correlated with methylation of the *RIL/CDH13* panel and negatively correlated with methylation of the *HIN-1/RASSF1A* panel.

Familial *BRCA1* and *BRCA2* tumors are associated with young age at onset and are phenotypically distinct from each other as well as from sporadic breast carcinomas [57]. Conventional histopathological and molecular analysis have demonstrated that familial *BRCA1* tumors predominantly have a basal-like phenotype and are significantly associated with certain features such as a negative ER and PR status, medullary tumor histological type, *TP53* mutations and high tumor grade [58]. Epigenetic silencing and deletion of the *BRCA1* gene in sporadic breast cancer could explain phenotypic similarities between *BRCA1* methylated and familial *BRCA1* breast tumors.

Predictors of therapy response

Next to DNA methylation predicting growth factor and steroid receptor status, Widschwendter *et al.* [54] found that methylation of *ER* and *CYP1B1* (encoding a tamoxifen/estradiol-metabolizing cytochrome P450) were both predictors of better disease-free survival (DFS) in patients treated with adjuvant tamoxifen, but not or to a lesser extent in patients who did not receive this type of therapy. This may imply a role of *ER* and *CYP1B1* methylation in tamoxifen efficacy. DNA methylation markers predicting response to endocrine therapy with tamoxifen in metastatic breast cancer have been described by our group [59]. Using a microarray containing CpG sites of the promoter regions of approximately 117 genes, DNA methylation of these genes was investigated in ER-positive primary tumors from 200 patients with metastatic disease. All patients received first-line endocrine therapy and had their response to this therapy recorded. Ten genes (*PSAT*, *STNM1*, *S100A2*, *SFN*, *PRKCδ*, *SYK*, *VTN*, *GRIN2D*, *TGFBR2* and *COX7A2L*) had a significant association

with therapy response. *PSAT*-promoter methylation displayed the strongest association and predicted a favorable response. Of those ten genes, a panel of five markers was extracted and patients with an adverse methylation signature based on those five genes had a 19% chance of responding to tamoxifen therapy compared with 74% of patients with a favorable profile (Figure 2). For *PSAT*, the strongest marker revealed in that study, promoter methylation predicted a favorable therapy response. Analysis of PSAT mRNA in the same specimen revealed the anticipated inverse association between DNA methylation of the gene and expression of its transcript. In line with this, we also observed that PSAT mRNA expression was associated with progression after endocrine treatment. Thus, PSAT behaves as a bonafide tumor suppressor of endocrine therapy response.

Outcome predictors

In breast cancer, approximately 70% of node-negative patients can be considered cured just by loco-regional treatment alone. However, established histopathological factors alone are not sufficient to identify these low-risk patients, and thus a considerable number of these patients receive adjuvant chemotherapy, which in fact constitutes a substantial overtreatment. We therefore searched for methylation markers that are associated with excellent outcome in node-negative, steroid receptor-positive breast cancer in order to identify those patients who would be sufficiently treated just by adjuvant endocrine therapy alone. Using a methylation microarray, 117 candidate genes were analyzed in steroid hormone receptor-positive tumors from 109 primary breast cancer patients treated with adjuvant tamoxifen. Results were then validated in an independent cohort (n = 236) from five centers. Independent methodological validation was achieved by a real-time PCR-based technique. DNA methylation of paired-like homeodomain transcription factor 2 (*PITX2*) showed the strongest correlation with distant recurrence, with 86% of patients with low *PITX2* methylation being metastasis-free at 10 years, compared with 69% with elevated *PITX2* methylation [60].

In a second multicenter, retrospective study these results were technically and clinically validated using PCR technology in FFPE tumor tissue [61]. First, methylation measurements in FFPE specimens and those in fresh–frozen specimens from the same tumor correlated reasonably well (r_s = 0.81; n = 89). Second, reproducibility of the PCR assay in replicate measurements in FFPE samples was very good (r_s > 0.95; n = 150). Last, but not least, regarding prognostic information for time to distant metastasis, *PITX2* methylation added significant independent information (hazard ratio [HR]: 2.35; 95% confidence interval [CI]: 1.20–4.60) to established prognostic factors (tumor size, grade and age) in a cohort of 399 hormone receptor-positive, node-negative breast cancer patients who received adjuvant tamoxifen monotherapy from ten clinical centers [61]. Given the results from the two marker discovery and validation studies [61], *PITX2* methylation analysis in clinically available FFPE samples may thus aid in clinical decision making as a practical assay to predict outcome in node-negative, tamoxifen-treated breast cancer and may allow the identification of those low-risk patients who may be sufficiently treated by endocrine therapy alone.

However, predictors of outcome determined in the primary tumor tissue of patients receiving adjuvant therapy can be associated with the innate aggressiveness of the disease or with response to the given therapy or with both. In a follow-up study [62] we therefore addressed whether *PITX2* DNA methylation is associated with distant metastasis-free survival in 412 lymph node-negative, steroid hormone receptor-positive breast cancer patients who had not received any adjuvant systemic treatment. *PITX2* DNA-methylation levels measured in DNA from tumor tissue using a real-time PCR-based assay was associated in uni- and multi-variate analysis with early distant metastasis and poor overall survival (for Kaplan–Meier curve see Figure 3). We thus concluded that *PITX2* is hypermethylated in aggressive breast cancer and therefore may serve – according to the definition – as a prognostic marker.

Another important subgroup of breast cancer that may also benefit from outcome predictors is patients with node-positive disease. These patients generally received adjuvant anthracycline-based chemotherapy, in particular if they have luminal subtype tumors – that is, ER-positive but HER2-negative tumors. However, the role of anthracyclines in HER2-negative disease is controversial [63]. Moreover, anthracycline-based chemotherapy is associated with a significant risk of late cardiotoxicity, and new anthracycline-free, taxane-based regimens have emerged [64]. We therefore tested the significance of *PITX2* methylation to predict outcome in 241 node-positive luminal breast cancers who received adjuvant anthracycline-based chemotherapy [65]. Also in this cohort, *PITX2* methylation predicted poor outcome. Thus, *PITX2* DNA methylation

seems to be a general predictor of poor outcome in luminal breast cancer independent of lymph node involvement and independent of whether adjuvant endocrine and/or conventional chemotherapy or no adjuvant systemic therapy at all had been administered. In the node-positive cohort, next to *PITX2*, we screened 60 additional candidate genes either identified by genome-wide discoveries for prognostic markers or because they were members of the *PITX2* pathway, with the aim to identify other DNA methylation markers predicting outcome in this cohort. A total of 15 of these genes were predictors of outcome, and among them we found many from the *PITX2* pathway, thus suggesting that DNA methylation of members of the *PITX2* pathway marks aggressive and/or potentially therapy-resistant disease. A four-marker panel including *PITX2*, *BMP4*, *FGF4* and *C20orf55* showed improvement of outcome prediction over conventional predictors and over *PITX2* as a single marker. Thus, these results suggest that a well-defined panel of DNA methylation markers enables clinically relevant outcome prediction, even in lymph node-positive, HER2-negative breast cancer patients treated with anthracycline-based chemotherapy. A summary of all studies evaluating the clinical usefulness of *PITX2* in primary breast cancer can be found in Table 1.

To conclude, currently available evidence regarding hypermethylated target genes as potential prognostic and predictive factors in breast cancer suggests that a 'methylation signature' or multiple-gene methylation panel analysis will be useful in improving the sensitivity and specificity of outcome prediction. Epigenomic profiles such as prognostic dendograms have been developed similar to those used in expression microarray analyses. These dendograms are complementary to gene-expression patterns and genetic alterations, with the advantage that they can be assayed using DNA from archived materials [31].

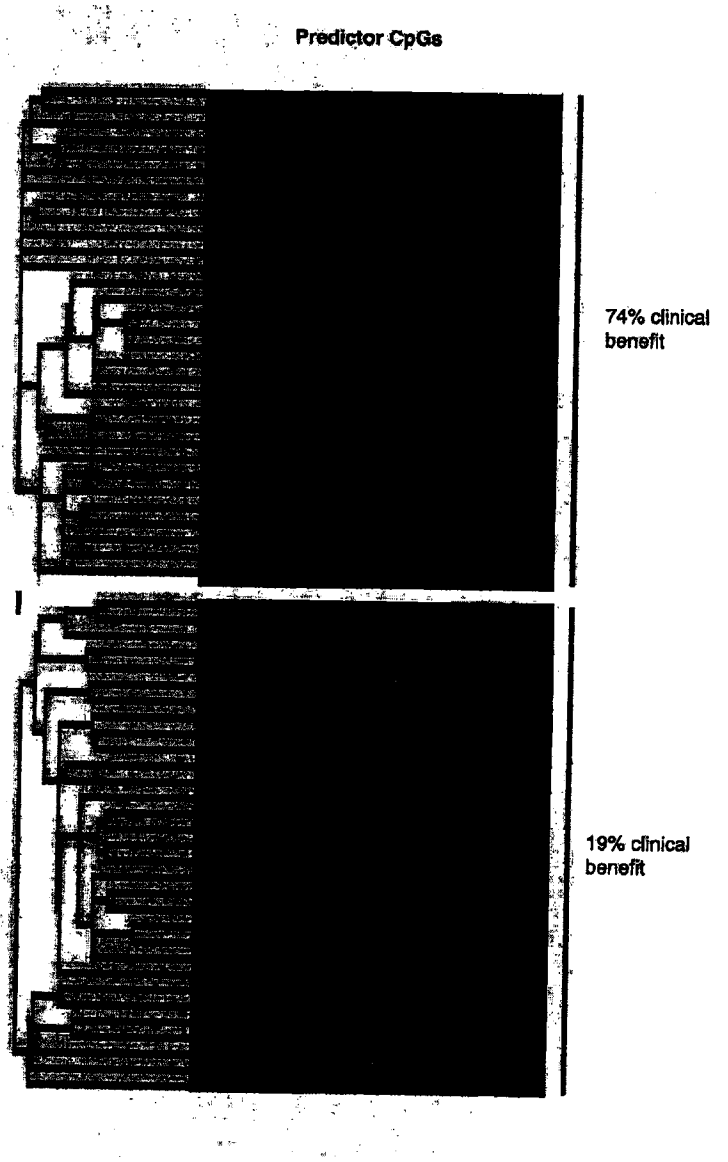

Figure 2. DNA-methylation marker-based risk stratification of metastatic breast cancer patients treated with first-line tamoxifen monotherapy. Unsupervised clustering, performed using the CpG methylation status from the genes that have independent information, that is, *PSAT1*, *STMN1*, *S100A2*, *GRIN2D* and *TGFBR2*, identified two clusters with a clear differential response to the endocrine treatment (tamoxifen) administered as a first-line therapeutic to the metastatic breast cancer patients included in the study. Rows represent patients/tumors and columns represent CpG sites. Red and green represent relative hyper- and hypomethylation, respectively.

DNA methylation biomarkers for monitoring cancer therapy & disease progression

There are some preliminary data on the possibility of monitoring efficacy of adjuvant therapy in cancer patients by methylation analysis. For example, Fieghl *et al.*, using cir-DNA, reported the disappearance of *RASSF1A* DNA methylation in serum during adjuvant tamoxifen treatment as a response indicator, whereas persistence or *de novo* appearance of *RASSF1* DNA methylation correlated with resistance to the same treatment [66]. In a study on *APC* and E-cadherin gene promoter hypermethylation using cir-DNA of serum and tumor tissue DNA from breast cancer patients, Zhang *et al.* showed that aberrant methylation of serum DNA can disappear after breast cancer surgery, implying that this approach may be useful in evaluation of therapy response and monitoring of breast cancer recurrence [67]. However, controlled clinical

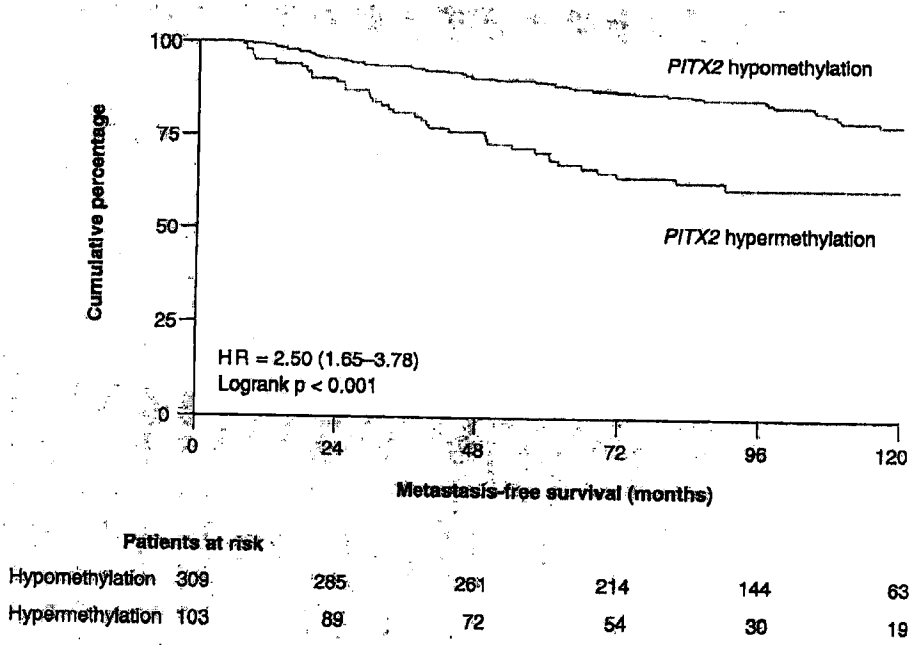

Figure 3. Kaplan–Meier analysis for metastasis-free survival dichotomized based on *PITX2* DNA methylation. Kaplan–Meier survival curves for MFS are presented for 412 lymph node-negative steroid hormone receptor-positive patients who did not receive any adjuvant systemic therapy. Patients were divided in four quartiles by the *PITX2* DNA-methylation rate. The first three quartiles showing hypomethylation were combined and compared with the fourth quartiles showing hypermethylation. MFS: Metastasis-free survival; PITX2: Paired-like homeodomain transcription factor 2. Reanalysis of data from [62].

trials addressing these issues are still lacking. It thus remains to be seen whether modifications made in adjuvant therapeutic strategies based on detection of cir-DNA methylation will improve patient survival as well as quality of life. Brock *et al.* studied methylation in an eight-gene panel in malignant pleural effusions combined with cytological examination [68]. Cytology alone had a 63% sensitivity, whereas methylation alone had a sensitivity of 67% for confirming diagnosis of malignant pleural effusion. If cytology and methylation were considered together, sensitivity reached 88% and specificity 100% in discriminating benign from malignant effusions. Thus, methylation analysis complements cytology and improves the diagnostic accuracy of the currently available tests of pleural fluid assessment for the presence of malignancy.

Table 1. Clinical impact of *PITX2* methylation in primary breast cancer: retrospective clinical studies.

Study (year)	n (centers)	Setting	PITX2 assay	Significant prognostic impact	Ref.
Maier *et al.* (2007)	109 (1)	N^+, N0, all HR^+, TAM monotherapy	Microarray, fresh–frozen tissue	Yes	[60]
Maier *et al.* (2007)	236 (5)	All N0 and HR^+, TAM monotherapy	QM-PCR, fresh–frozen tissue	Yes	[60]
Nimmrich *et al.* (2008)	412 (1)	All N0 and HR^+, no adjuvant therapy	QM-PCR, fresh–frozen tissue	Yes	[62]
Harbeck *et al.* (2008)	399 (10)	All N0 and HR^+, TAM monotherapy	QM-PCR, FFPE tissue	Yes	[61]
Hartmann *et al.* (2009)	241 (4)	All N^+, $HER2^-$, HR^+, adjuvant anthracycline-based chemotherapy	Microarray, fresh–frozen tissue	Yes	[65]

FFPE: Formalin-fixed, paraffin-embedded; HR: Hormone receptor status; N0: Node-negative; N+: Node-positive; PITX2: Paired-like homeodomain transcription factor 2; QM: Quantitative methylation; TAM: Tamoxifen.

Conclusion & future perspective

As summarized in this review, DNA methylation is an emerging field of biomarkers. Promising results highlight its potential for early detection, therapy monitoring, assessment of prognosis and prediction of therapy response in patients with early and with advanced breast cancer. DNA provides a very stable and robust analyte, which is therefore particularly suitable for clinical applications since it can be determined in various clinical materials processed under routine conditions. In order for methylated gene markers to enter routine clinical use, simple, rapid, quantitative, accurate, standardized and cost-effective assays, which are applicable to a variety of tumor types and sources of DNA, will be required [1,14]. Moreover, their clinical value needs to be validated in high-level evidence studies, such as prospective trials [2]. Although potentially useful, up until now only *PITX2* DNA methylation has been validated in a large, independent, multicenter cohort using a validated assay with a prospectively determined cut-off value. All other DNA methylation biomarkers mentioned here are still in the first phase of development and will still need to be independently validated before any clinical application. Regarding *PITX2* DNA methylation, in order to achieve the highest level of evidence for allowing it to be used in the clinical routine, prospective validation is needed, preferably comparing it in a randomized trial to standard of care. After successful prospective validation, the biomarker *PITX2* may be used alone or in combination with other prospective validated biomarkers that predict outcome in hormone receptor-positive lymph node-negative breast cancer patients receiving adjuvant endocrine therapy. Using this validated information, the clinician may be able to advise a patient with a favorable profile to receive endocrine therapy as the only adjuvant systemic treatment, since for such a patient this generally well-tolerated therapy would be sufficient to guarantee long-term survival.

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No writing assistance was utilized in the production of this manuscript.

Executive summary

- DNA methylation is a frequent and early event in cancer.
- DNA methylation occurs in mammals only at cytosines on the fifth residue of its base, and this methylcytosine nucleotide is often referred to as 'the fifth base'. Cytosine–phosphate–guanosine (CpG) methylation negatively affects gene transcription of an adjacent gene.
- DNA methylation has interesting distinct features that suggest its use as a cancer biomarker: first, DNA methylation is relatively stable compared with most other biomarkers, and accessible from routinely collected formalin-fixed paraffin-embedded (FFPE) clinical material. Second, there are numerous potential human DNA sources.
- Cancer-specific DNA hypermethylation patterns have been detected in FFPE or fresh–frozen tumor specimens, exfoliated luminal tumors cells, cellular DNA and free-tumor DNA from plasma, ductal lavage from the breast, and other body fluids such as urinary sediment, saliva, sputum, bronchial washings and ejaculate.
- Breast cancer is a very prevalent human malignancy and a common cause of cancer-related death among women worldwide.
- Novel biomarkers are needed in breast cancer for outcome prediction, particularly in node-negative disease. Thus far, among novel biomarkers, only urokinase plasminogen activator and plasminogen activator inhibitor-1 have been validated at the highest level of evidence for therapy decision making in early breast cancer.
- Regarding screening, no suitable gene or set of genes consistently hypermethylated in breast cancer but not in healthy tissue and detectable in serum or plasma have yet been found.
- Regarding outcome prediction, only paired-like homeodomain transcription factor 2 (*PITX2*) DNA methylation has been validated in early breast cancer in a large, independent, multicenter cohort using a validated assay with a prospectively determined cut-off value: *PITX2* methylation analysis in clinically available FFPE samples thus may aid in clinical decision making, and allow to identify those low-risk node-negative breast cancer patients who may be sufficiently treated by adjuvant endocrine therapy alone.
- In order to be implemented in clinical practice, next to clinically validated data, simple, rapid, quantitative, accurate, standardized and cost-effective methylation assays, which are applicable to a variety of tumor types and sources of DNA, will be required.

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IV. Scientific Publications

1: Tänzer M, Balluff B, Distler J, Hale K, Leodolter A, Röcken C, Molnar B, Schmid R, Lofton-Day C, Schuster T, Ebert MP. Performance of epigenetic markers SEPT9 and ALX4 in plasma for detection of colorectal precancerous lesions. PLoS One. 2010 Feb 4;5(2):e9061. PubMed PMID: 20140221; PubMed Central PMCID: PMC2816214.

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V. Presentations at Scientific Meetings

92nd Annual Conference of the German Pathology Society e.V.
- Week of Pathology - Berlin, Germany (2008)
Poster: "Early detection of prostate cancer using DNA methylation analysis of the GSTP1 gene in histopathologically negative prostate cancer specimens: potential to improve the clinical routine?"

CNAPS-V Conference, Moscow, Russia (2007)
Presentation: "Septin 9 Methylation as a Plasma Biomarker for Colorectal Cancer"

Distler, J. (1991) Streptomycin biosynthesis and its regulation in *Streptomyces griseus*. International Symposium on the Biology of Actinomycetes, ISBA´91; University of Wisconsin-Madison, USA.

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VI. Selected Patents and Patent Applications

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